

**The efficacy and function of commercial *Rhizophagus irregularis* inoculum for wheat growth in a changing climate**

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## Abstract

Arable agriculture urgently requires sustainable solutions to reduce reliance on large inputs of nutrient fertilisers to continue to improve crop yields. By harnessing the symbiosis between arbuscular mycorrhizal fungi (AMF) and plants, there is potential to enhance plant nutrient assimilation and growth with fewer additional inputs, such as chemical-based fertilisers. However, the efficacy of commercially-available mycorrhizal inocula in agricultural systems remains controversial. There is a pressing need to assess the functional significance of AMF inocula in crops across cultivars to successfully exploit AMF in agriculture. Moreover, climate change is now negatively impacting agricultural productivity, and we know little about how increasing atmospheric [CO<sub>2</sub>] will change the functionality of mycorrhiza-crop relationships.

Using isotope tracers (<sup>14</sup>C, <sup>33</sup>P and <sup>15</sup>N), I measured carbon-for-nutrient exchange between wheat and AMF symbionts with- and without the addition of a commercially available active mycorrhizal inoculum (*Rhizophagus irregularis*) to a non-sterile agricultural soil to simulate in-field application scenarios. I quantified whether the response to *R. irregularis* inoculum was cultivar-specific using three elite wheat cultivars in common use in rotations today. Inoculation with AMF increased phosphorus uptake across all wheat cultivars, although the increase was not directly attributable to mycorrhizal fungi and there were significant cultivar differences in carbon-for-nutrient exchange stoichiometry.

Using T-RFLP to analyse the community composition in wheat roots, I found *R. irregularis* introduced via inoculation established in the soil and successfully colonised all cultivars. This suggests there is an increased likelihood that any responses of the wheat cultivars to inoculation were directly due to *R. irregularis* colonisation and associated rhizobacteria rather than any facilitation effects on the native AMF community.

AMF functioning can be highly context dependent and strongly driven by environmental factors. Therefore, due to the increasing interest in the application of commercially available AMF inoculants in agricultural soils, it was important to quantify changes in carbon-for-nutrient exchange between the cultivars and *R. irregularis* at elevated atmospheric [CO<sub>2</sub>] (in line with IPCC projections for atmospheric CO<sub>2</sub> concentrations in 2100). I found no evidence for enhanced

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carbon-for-nutrient exchange at elevated [CO<sub>2</sub>], addressing a critical knowledge gap of how fungal inoculants, which could function well in current systems, will respond to future climates.

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## List of Abbreviations

[CO<sub>2</sub>] = Atmospheric carbon dioxide concentrations

AM = Arbuscular mycorrhiza

AMF = Arbuscular mycorrhizal fungi

C = Carbon

ERM = Extra-radical mycelium

FACE = Free-air carbon dioxide enrichment

GHG = Greenhouse gas

gs = Stomatal conductance

IRM = Intraradical mycelium

ISR = Induced systemic resistance

N = Nitrogen

N<sub>2</sub> = Atmospheric nitrogen

NH<sub>3</sub> = Ammonia

NH<sub>4</sub> = Ammonium

NO<sub>3</sub> = Nitrate

NUE = Nutrient use efficiency

P = Phosphorus

P<sub>i</sub> = Orthophosphate

PPM = Parts per million

PSB = Phosphorus solubilising bacteria

RuBisCO = Ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBP = Ribulose-1,5-bisphosphate

T-RF = Terminal restriction fragment

T-RFLP = Terminal restriction fragment length polymorphism

WUE = Water use efficiency

## **Chapter 1 General Introduction**

### **1.1 Food security and sustainable agriculture**

#### **1.1.1 Food security**

When the human population first reached 1 billion in 1800, it continued to increase at a slow and steady rate until the 1950s when it reached 2.5 billion (Bongaarts, 2009). Since then, growth has accelerated at historically unprecedented levels and will reach ten billion by 2050. This exponential growth rate is a result of a declining death rate after the industrial revolution driven by a decline in crisis mortality (e.g. epidemic diseases or famine), due to better living conditions and health provisions such as vaccinations. In the northern hemisphere, birth rates have started to fall, but in the southern hemisphere, where many countries are still developing, population growth continues to increase (Cohen, 2003).

The rising demand for food from the growing human population has driven a doubling of grain harvests over the past fifty years. This substantial increase in food production is referred to as the 'Green Revolution' and was a result of several factors, including the development and application of chemical fertilisers, novel pesticides and irrigation, together with breeding of high-yielding crop cultivars (Foley, 2005). These new cultivars, with a dwarfed phenotype, led to astounding increases in wheat and rice yields during the Green Revolution and are now known as semi-dwarf varieties (Hedden, 2003). The genes responsible for dwarfed growth disrupt the plant's ability to produce or detect the gibberellin growth hormone (Sasaki et al., 2002; Peng et al., 2013). Previously, the stems of cereal crops were unable to support grain weight when plants produced high yields, leading to stem lodging and yield losses. Semi-dwarf varieties produced shorter stronger stems, allowing greater application of nutrient fertilisers, without fear of the ear biomass causing stem collapse (Hedden, 2003). After the 1960s, the use of nutrient fertilisers has increased by 700% and contributed significantly to agricultural output (Foley, 2005). We now release as much N and P into the terrestrial environment through the use of agricultural fertilisers as all-natural processes combined (Tilman et al., 2001). For example, natural N fixation on land

was estimated to be up to 130 Tg y<sup>-1</sup>, and human activity including fertiliser additions, cultivation of legumes and fossil fuel combustion was estimated to add over 140 Tg y<sup>-1</sup> and is continually increasing (Vitousek et al., 1997).

Simultaneously, the formulation of new insecticides, herbicides and fungicides from the 1960-80s significantly improved pest control (Aktar et al., 2009). Without pesticides, up to 70% of crop yields would be lost to pests (Oerke, 2006). Of these pests, weeds (i.e. non-target plants in competition with cultivated plants) have the most detrimental effect on yields with potential losses of up to 34%, mainly due to competition for inorganic nutrients. Animal pests and pathogens have the potential to cause losses of 18% and 16% respectively. Globally, farmers apply three million tonnes of chemical pesticides to crops every year (Popp & Hantos, 2011).

Despite increases in food production, in 2016, 11% of the world's population was still undernourished due to insufficient caloric intake (FAO, 2017). Predictions indicate we need to double food production again by 2050 to ensure continued food security, leading to calls for a second Green Revolution (Tilman et al., 2011). However, in many countries, including the UK, increases in yields of major crops have been stagnating (Ray et al., 2012). The UK's most important food crop is wheat, which is grown on over 40% of the land under arable cultivation (Rial-Lovera et al., 2017), but yield increases have ceased in 99% of harvested areas in the UK (Ray et al., 2012). Wheat provides 20% of the calories to the world's population and demand for wheat is expected to rise continuously up to 2050 by 1.7% annually, with growth in productivity currently unable to keep up (Reynolds et al., 2012).

Major crop yield improvements are traditionally achieved through breeding new elite cultivars. Artificial selection is used to screen hundreds of wheat specimens and only those with the best combination of desirable characteristics are selected for breeding into the next generation; this continues until no undesirable traits remain (Breseghello & Coelho, 2013). Traits which are selected for include yield, height, ear emergence and resistance to diseases and environmental stressors. Marker-assisted selection (MAS) allows earlier detection of desirable traits by linking them to genetic markers, therefore bypassing the need to phenotype plants (Collard & Mackill, 2008). MAS is highly advantageous especially for traits which are difficult to measure or are expressed late in development.



The Wheat Genetic Improvement Network developed a reference population from wheat cultivars Avalon and Cadenza to aid the discovery of new trait markers for breeding (Ma et al., 2015). These cultivars were chosen due to differences in their canopy architecture. Avalon carries recessive vernalization response genes (VRN) associated with the winter wheat growing habit, whereas Cadenza carries the dominant *Vrn-A1a* allele (Griffiths et al., 2009). The parents also have different alleles for reduced height semi-dwarfing genes (*Rht-D1*), with Avalon carrying the dwarf allele creating a shorter phenotype and Cadenza carrying the wild type allele (Griffiths et al., 2012). The Avalon x Cadenza population has been used to study a range of traits such as plant height (Griffiths et al., 2012), flowering time (Griffiths et al., 2009), root system traits (Bai et al., 2013) and resistance to diseases (Bass et al., 2006; Ma et al., 2015), leading to a high-density genetic map of trait markers which are useful for breeding. Cadenza contains the most positive markers for root traits and generally has a larger root system than Avalon (Ma et al., 2015). Cadenza also contains desirable markers for major resistance alleles *Yr6* and *Yr7* and mosaic disease resistance loci *Sbm1* (Ma et al., 2015).

MAS was used to help develop the UK's most widely grown wheat cultivar Skyfall (Allen-Stevens, 2019). Skyfall is a high yielding winter bread wheat variety which was released in 2014. Skyfall's success is due to its high-quality milling grain (National Association of British and Irish Millers group 1), good all-round foliar disease resistance and flexible sowing window from September to February (RAGT seeds, 2018).

Unless a substantial increase in agricultural productivity is achieved through breeding new elite cultivars or other means, it will be essential for new land to be brought into production (IPCC, 2018). However, converting more land to agricultural use would result in adverse environmental impacts, including large amounts of greenhouse gas (GHG) emissions, loss of biodiversity and socially, culturally and economically important wild green space (IPCC, 2018). If past trends continue, for example, developing countries continue to increase crop production mainly through land clearing, and more prosperous countries through farming intensification and yield improvement, 1 billion ha more land will need to be cleared by 2050 and result in 250 Mt  $y^{-1}$  of additional CO<sub>2</sub> emissions (Tilman et al., 2011). However, countries with the highest crop production are up to 308%

higher than the lowest yielding countries, so, investment in technological improvements, adaptation and transfer to developing countries could markedly reduce land clearing and environmental impacts (IPCC, 2018). Nevertheless, we also need to consider that achieving yield increases is likely to be further exacerbated by climate change.

### **1.1.2 Climate change and agriculture**

Human-induced warming reached approximately 1°C above pre-industrial levels in 2017, increasing at 0.2°C per decade (IPCC, 2018). Corresponding atmospheric CO<sub>2</sub> concentrations ([CO<sub>2</sub>]) have been rising at 20 ppm (parts per million) every decade, which is faster than any rate seen in the past 800,000 years (Lüthi et al., 2008). Rising temperatures have caused increases in unstable weather patterns such as periodic drought, flooding and heat-waves, with low to middle-income countries being most affected (IPCC, 2018). These countries contain a higher proportion of subsistence farmers who are heavily reliant on agriculture production, which is hugely vulnerable to temperature increases and shifting precipitation patterns (Shiferaw et al., 2014; Miyan, 2015). For example, in Syria a severe drought between 2007 and 2010 resulted in a sharp decline in agricultural productivity and displaced hundreds of thousands of people (Kelley et al., 2015).

Research suggests that crop losses due to climate change could be in part mitigated by increases in [CO<sub>2</sub>], which may increase crop production through enhanced photosynthesis and water use efficiency (WUE) (Rosenzweig & Parry, 1994), particularly, in plants which photosynthesise by C<sub>3</sub> carbon fixation, which uses the enzyme RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase). RuBisCO catalyses either the carboxylation or oxygenation of ribulose-1,5-bisphosphate (RuBP) during either photosynthesis or photorespiration. Photosynthesis is increased by elevated [CO<sub>2</sub>] due to increased carboxylation of RuBisCO while inhibiting oxygenation of RuBP in photorespiration (Drake et al., 1997). C<sub>4</sub> plants may have evolved to reduce photorespiration by concentrating CO<sub>2</sub> within the leaf to the bundle-sheath cells where the Calvin cycle takes place, and therefore the efficiency of RuBisCO is not as affected by atmospheric [CO<sub>2</sub>] (Hatch, 2002).

Free-Air CO<sub>2</sub> Enrichment (FACE) experiments, to test the effect of increasing [CO<sub>2</sub>] on the growth of crop plants, have shown that wheat yields have the

potential to increase by up to 9% in elevated [CO<sub>2</sub>] (550 ppm) (Tubiello et al., 1999), and 10% in 645 ppm [CO<sub>2</sub>] (Manderscheid & Weigel, 2007). Although, FACE experiments have also shown that acclimation of the photosynthetic machinery can occur and lead to the downregulation of photosynthesis after extended periods of exposure to elevated [CO<sub>2</sub>] (Ainsworth & Long, 2005). Additionally, any potential yield increases are caveated by faster plant growth rates brought upon by elevated [CO<sub>2</sub>], which can result in lower protein contents in the cereal grains (Myers et al., 2014). Decreases in the nutritional value of cereal crops where they are staple foods jeopardies the nutrition-related health of millions of people.

Nevertheless, exploring the potential beneficial opportunities associated with climate change remains essential. For example, an increase in [CO<sub>2</sub>] is frequently associated with a decline in stomatal conductance (gs), with gs decreasing up to 40% when [CO<sub>2</sub>] is doubled (Eamus, 1991). Similarly, elevated [CO<sub>2</sub>] is related to lower stomatal densities; plasticity in stomatal development and behaviour could significantly improve WUE by decreasing transpiration. In C<sub>4</sub> crops such as maize, FACE experiments have demonstrated that yield benefits associated with elevated [CO<sub>2</sub>], are only present under water-limited conditions. In water-limited treatments, maize yields increased by 40% in high [CO<sub>2</sub>] treatments (Manderscheid et al., 2014). Likewise, in wheat, grain yield rose by only 10% in elevated [CO<sub>2</sub>] under well-watered conditions, but by over 40% in drought-stress conditions, indicating increasing [CO<sub>2</sub>] may ameliorate the adverse effects of summer drought on wheat grain yield (Manderscheid & Weigel, 2007).

However, future climate simulations have reported a decrease in global crop production with developing countries being the worst affected, despite increased [CO<sub>2</sub>] (Rosenzweig & Parry, 1994). For every 1°C rise in temperature models regularly predict decreases in crop production, across four principal crop cultivars wheat, rice, maize and soybean, yields would reduce by 6%, 3.2%, 7.4% and 3.1% respectively (Zhao et al., 2017a). Climate change impacts on agriculture and food prices could force millions of people into extreme poverty (Hallegatte & Rozenberg, 2017).

To avoid the disastrous consequences of climate change, we must limit warming to 1.5°C, meaning emission reductions must start immediately, and reach net-zero CO<sub>2</sub> emissions by 2050 (IPCC, 2018). Achieving net-zero emissions will

involve moving to renewable forms of electricity and substantially reducing emissions from agriculture. GHG emissions from agriculture, including both crop and livestock production, account for 30% of total anthropogenic emissions (IPCC, 2018). Of agricultural GHG emissions, a large proportion stems from fertiliser production, distribution and application, 16% from organic fertilisers and 13% from synthetic fertilisers (Tubiello et al., 2013).

### **1.1.3 Sustainable agriculture**

To achieve increases in food production while decreasing GHG emissions, more sustainable practices in agriculture must be implemented. Continued use of large volumes of nutrient fertilisers is particularly unsustainable. The concept of sustainable agriculture gained prominence in the 1980's; however, its meaning can be ambiguous (Velten et al., 2015). Most definitions of sustainable agriculture incorporate goals of producing adequate food to meet human demand while conserving resources, minimising environmental impact and maintaining profitability for farmers. Sustainable agriculture encompasses a wide array of management strategies, which are suitable for different environments and crops (Rockström et al., 2017). For example, organic farming systems replace chemical fertilisers and pesticides with organic fertiliser inputs and enhance pest control through ecosystem services (Azadi et al., 2011). However, organic farming systems often result in a yield penalty, often producing only 80% of conventional crop yields, although this is highly variable (De Ponti et al., 2012). Due to this yield penalty organic farming may not be sustainable, and meta-analyses which measure agricultural environmental impact per unit food produced have found organic systems cause more eutrophication, and have equal GHG emissions to conventional agriculture (Clark & Tilman, 2017). Conservation agriculture has been widely promoted among small-holder farms in Sub-Saharan Africa and promotes minimal soil disturbance through non-tillage, permanent soil cover through cover crops and diverse crop rotations (Pittelkow et al., 2015). Conservation agriculture aims to enhance below-ground biodiversity and nutrient cycling to increase water and nutrient use efficiency (NUE), which could be vital to try and reduce fertiliser use. Although the methods mentioned above could be incorporated into a sustainable agriculture system, no single strategy should be

promoted universally, and different methods need to be tailored to different crops and environments.

#### **1.1.3.1 Phosphorus-based fertilisers**

Phosphorus (P) is an essential nutrient for plant growth, but plant-available P in the soil is relatively scarce, making up just 1% of total soil P (Blake et al., 2000). Plant available P is in the form of negatively charged orthophosphate ( $P_i$ ) (Smith et al., 2011) and occurs in deficient concentrations in the soil because it is highly reactive with aluminium, iron and calcium, forming inorganic structures with low solubility. A large proportion of soil P is in organic forms, predicted to be between 20% and 80% dependent on soil type (Dalai, 1977). Plants may gain access to organic forms of P when microorganisms mineralise them, this includes some evidence that arbuscular mycorrhizal fungi can mineralise organic P compounds through enzyme release (Koide & Kabir, 2000).

Due to the low levels of plant-available P in the soil, agriculture is highly reliant on the application of inorganic P fertilisers, acquired through mining rock phosphate (Cordell et al., 2009). Rock phosphate is a non-renewable resource and estimates predict global reserves will be exhausted within the next 30-300 years (Cordell et al., 2009; Cordell & White, 2013). Demand for P is expected to double by 2050; however, the majority of phosphate rock reserves are in Morocco, China and the USA, with Europe completely dependent on imports (Cordell et al., 2009). Therefore, P use in agriculture needs to change dramatically, and resources must be used efficiently, recycled and supplemented with alternatives.

#### **1.1.3.2 Nitrogen-based fertiliser**

Nitrogen (N) is a critical element of nucleotides and proteins and therefore is essential for life. In natural systems, N availability in the soil often limits plant growth (Robertson & Vitousek, 2009). Thus, the addition of N fertilisers is now a fundamental part of agricultural systems. Nitrogen-based fertilisers are produced via an energy-intensive method called the Haber-Bosch process (Haber et al., 1912). N gas is made up of two atoms which share a strong triple covalent bond; in nature, the energy required to break these is only achieved by powerful lightning storms. Nitrogen-fixing microbes with enzymes capable of catalysing the conversion atmospheric nitrogen ( $N_2$ ) to ammonia ( $NH_3$ ) occur both in the soil and in specialised plant roots (mostly leguminous plants). The Haber-Bosch process

turns  $N_2$  into  $NH_3$ , via a reaction with hydrogen, requiring high temperature (375-475°C) and pressure (50-200 bar) (Vojvodic et al., 2014). However, the Haber-Bosch process is essential for food production, and estimates suggest that by producing N in a plant-available form for application as fertiliser, it has fed over 40% of people born since 1908 (Erisman et al., 2008).

Over 80% of N fertilisers applied to soil in the USA is lost from agricultural systems, causing extensive environmental changes to natural ecosystems (Robertson & Vitousek, 2009). For example, freshwater deposits containing high levels of N have created a dead zone in the Mississippi basin of over 20,000 km<sup>2</sup> (Rabalais et al., 2002). The dead zone is caused by excessive algal growth, which when decomposed by bacteria consumes all dissolved oxygen within the system, leading to a hypoxic zone. Alternatively, N can leach into the groundwater supply, making it unsuitable for human consumption, or be lost to the atmosphere as various N-containing gases including  $N_2O$ , a 300 times more potent GHG than  $CO_2$  (Robertson & Vitousek, 2009). A large proportion of the GHGs created during food production are a result of manufacturing N fertilisers. As countries aim to curb climate change and improve food security, sustainable nutrient management strategies, which aim to reduce our reliance on high nutrient inputs in agriculture, are sorely needed.

## **1.2 Arbuscular mycorrhizal fungi**

Arbuscular mycorrhizal fungi (AMF) form associations with >70% of vascular plant species (Brundrett & Tedersoo, 2018). This widespread association is one of the most ancient plant symbioses dating back to the evolution of terrestrial plants up to 500 million years ago (Corradi & Bonfante, 2012; Morris et al., 2018).

AMF are grouped in the monophyletic phylum Glomeromycota (Schüßler et al., 2001). Traditionally, taxonomy was based on spore morphology which led to the dominance of one genus, *Glomus* (Young, 2012). However, reliable identification based on spore morphology is extremely difficult as there are very few distinguishing features and within one culture, spores can vary in size and colour. Similar spore morphology has now been identified in multiple clades of the Glomeromycota (Schwarzott et al., 2001). Molecular comparisons between species using three rDNA regions (SSU, LSU, and ITS) from >100 named species has led to almost all species previously known as *Glomus* being designated to a

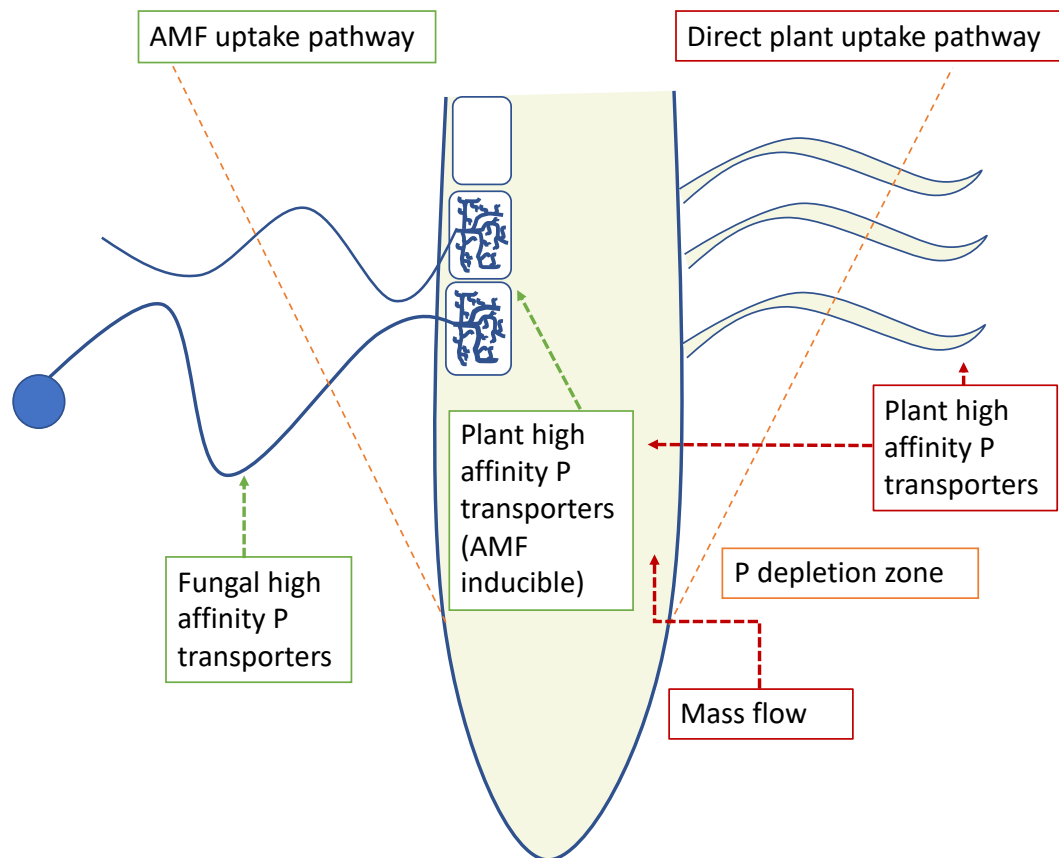
new genus (Schüßler and Walker, 2010; Krüger et al., 2011). Even the model species of AMF, known at the time as *Glomus intraradices*, was wrongly classified (Stockinger et al., 2009). Phylogenetic analysis of multiple isolates of *G. intraradices* revealed that the isolates separated into two clades. Model isolates including DAOM197198 and BEG195 were not *G. intraradices* and aligned with a newly described species *G. irregulare* (Stockinger et al., 2009), now known as *Rhizophagus irregularis* (Schüßler and Walker, 2010). Due to the large number of species name changes between current and older studies in the literature, both current and former names will be indicated when first mentioned within this thesis.

As AM fungus colonises plant roots, it produces intracellular tree-like nutrient exchange sites known as arbuscules within the cortical cells of plant roots. AMF hyphae can far outperform the ability of roots to access nutrients due to their ability to proliferate through large soil volumes beyond the nutrient depletion zone surrounding the roots, which is created as the roots uptake nutrients from the soil faster than the nutrients can be replaced via diffusion. In addition, hyphae are much finer than roots allowing them access to soil pores unreachable via the root system (Smith et al., 2011). Plants can allocate high levels of carbon (C) to their fungal partner, between 4% and 20% of what they produce through photosynthesis (Douds et al., 1988; Jakobsen & Rosendahl, 1990). However, recent evidence suggests plant carbon allocation to AMF may be lower than previously estimated and frequently falls below 10% of the plant C budget (Konvalinková et al., 2017). In return plants may receive between 0 % and 80% of required P and N through their fungal partner (Smith & Read, 2008). If AMF can make a substantial contribution to plant P and N uptake and can be exploited successfully in agricultural systems, they could lead to a reduced reliance on fertilisers to maintain crop yields (Cozzolino et al., 2013).

### **1.2.1 AMF and plant nutrition**

Arbuscular mycorrhizal (AM – i.e. the symbiosis as a whole referring to the plant and the fungus) plants have two pathways by which they can acquire nutrients, either directly through their root hairs and epidermis, or via a fungal symbiont (Figure 1.1). The AM pathway involves the uptake of soil nutrients by the fungal mycelium, translocation along the hyphal network to the intraradical fungal structures and transfer of nutrients to the plant cell (Smith & Smith, 2011). The extraradical mycelium has high-affinity inorganic phosphate (Pi) transporters for

acquiring Pi from the soil; Pi passes to the plant across a symbiotic interface, called the periarbuscular membrane (Harrison, 2002). The mechanism by which AMF efflux Pi into the apoplast is unknown, but Pi uptake into the root cells involves transporters which are expressed explicitly in colonised cortical cells (Paszkowski et al., 2002; Glassop et al., 2005; Christophersen et al., 2009). NH<sub>4</sub><sup>+</sup> Transporters are also preferentially expressed in the periarbuscular membrane, indicating N transfer to the plant may occur similarly (Guether et al., 2009).



**Figure 1.1: The direct and mycorrhizal phosphorus (P) uptake pathways in a root with arbuscular mycorrhizal fungi (AMF) associations.** The direct pathway involves high-affinity P transporters located in root hairs and epidermal cells. P uptake by the direct pathway results in a depletion zone close the roots. The mycorrhizal pathway involves P uptake by fungal high-affinity P transporters, translocation along the extra-radical hyphae and transfer into the root via the symbiotic interface. This involves AMF inducible P transporter (adapted from Smith and Read 2008).



For many years it was assumed that P uptake via the AM pathway and the direct pathway were additive (Smith & Read, 2008). However, experiments using radio-labelled P suggest that the AM pathway is commonly the primary uptake pathway for P; in some cases, it is responsible for almost all P uptake (Smith et al., 2004). Substantial proportions of P uptake through the mycorrhizal pathway occur, even in systems where there is no increase in whole-plant P content, implying downregulation or elimination of the direct pathway in some AM plants (Li et al., 2008; Smith et al., 2009, 2011). Reduced P uptake by the direct pathway may be due to a P depletion zone in the rhizosphere caused by gradual depletion of Pi concentrations in the soil, where absorption is faster than replacement by diffusion or mass flow, the depletion zone is extended by the extra-radical mycelium past the rhizosphere (Smith & Smith, 2011). Competition for Pi in the depletion zone between root and AMF could be responsible for the depression of the direct uptake pathway if AMF are more effective competitors for Pi. Alternatively, the downregulation of P transporter genes within plants has been hypothesised to explain these changes to the direct uptake pathway in response to associations with AMF (Grønlund et al., 2013).

Compared to P, inorganic forms of N are relatively mobile in the soil and transported to the roots by mass flow, so depletion zones are not considered a significant issue unless the land is dry (Smith & Read, 2008). Plants and AMF can absorb both nitrate ( $\text{NO}_3$ ) and ammonium ( $\text{NH}_4$ ), but AMF contribution to plant N uptake, has received much less attention than plant P uptake. It is now evident that the AM pathway can make contributions to N uptake from the soil, but the relative importance of the pathway in terms of total N uptake is still uncertain (Smith & Smith, 2011), considering that plants need about ten times more N than P.

Experiments involving multiple plant-fungal combinations have demonstrated forms of both  $^{15}\text{NO}_3$  and  $^{15}\text{NH}_4$  can be transferred to plants by their AMF partners from a compartment with only hyphal access (Ames et al., 1983; Tobar et al., 1994; Hawkins et al., 2000; Mäder et al., 2000; Hawkins & George, 2001; Thirkell et al., 2016). Some studies showed high contributions of the AMF pathway to N uptake, for example 42% of plant N was attributed to hyphal uptake in tomato (Mäder et al., 2000), and monoxenic cultures of carrot roots gained around 30% of N in the roots from *G. intraradices* (now *Rhizophagus irregularis*)

(Govindarajulu et al., 2005). However, often N transfer to the plant by AMF did not result in increased total N content or growth of the plant (Ames et al., 1983; Hawkins et al., 2000; Mäder et al., 2000; Reynolds et al., 2005)

### **1.2.2 Non-nutritional benefits**

The majority of research into the AM symbiosis has concentrated on the ability to relieve nutrient limitations encountered by plants. However, beyond nutrient exchange, AMF may contribute to sustainable agriculture through a range of mechanisms. For example, AMF play a crucial role in soil structure and health, mainly through their contributions to forming and stabilising soil aggregates (Rillig, 2004; Leifheit et al., 2014). Improved soil structure can prevent erosion and enhance C storage in the soil, which increases water infiltration in the soil and water holding capacity. AMF secure soil aggregates directly through their high-density hyphal networks, which act as support holding together soil aggregates, and indirectly through changes to root architecture and plant water regimes (Rillig, 2004; Leifheit et al., 2014). The AMF can increase soil water retention and C concentrations, both of which are essential for soil function and plant growth (Daynes et al., 2013).

There is strong evidence that AMF colonisation affects plant water relations and improves drought tolerance (Augé, 2001). However, it is often difficult to distinguish between effects which are due to fungal colonisation and effects which occur indirectly due to changes in plant growth and nutrient status. For example, the impact of AMF on stomatal conductance and transpiration rate has been observed regularly in studies between AM and non-AM plants, more commonly observations indicate that AMF increase stomatal conductance rates by an average of 24%, however, effects are unpredictable and not always apparent (Augé et al., 2014). There is also an interplay between stomatal conductance and P status, in studies where P nutrition was improved stomatal conductance increased by a much higher average of 52%. Also, both AMF and increased transpiration have been shown to alter root architecture, plants with higher stomatal conductance had an increase in root hair length and density, presumably to counteract water loss (Hepworth et al., 2016). AMF can change root architecture in terms of root length, number of lateral roots, branching and density of roots (Wu et al., 2013). These changes to root system architecture

allow mycorrhizal plants to explore a larger soil volume and thus, enhance plant drought tolerance.

A further non-nutritional benefit gained by associating with AMF is the plant's increased ability to defend against pests and diseases. Mycorrhizal associations have bio-protective qualities against many plant pathogens, both above- (Campos-Soriano et al., 2012) and below-ground (Vos et al., 2012). AMF association with roots triggers a transient rise in plant defences, which causes a temporary defence response within the plants salicylic acid and jasmonic acid signalling pathways, later suppressed by the symbiont (Kapulnik et al., 1996). AMF may employ strategies similar to those of pathogenic fungi to suppress plant immune responses, for example *R. irregularis* has been shown to secrete an effector protein (SP7), which when translocated to the plant nucleus reduces a pathogenesis-related transcription factor (ERF19) known to be triggered by fungal pathogens and only transiently expressed upon colonisation with AMF (Kloppholz et al., 2011). This initial induction of defences 'primes' the plant's immune system for future pathogen invasions, inducing a stronger and faster defence response, often referred to as Induced Systemic Resistance (ISR) (Conrath et al., 2007). Additionally, AMF can improve tolerance to herbivore attacks directly through enhancing plant defence capacities through ISR and indirectly through improving plant nutrition (Hoffmann et al., 2011). However, the multitrophic interactions between AMF, plants and herbivores are not always beneficial to the plant. Insects which are specialised to a certain plant host and have a degree of resistance to their hosts defences perform better on AM plants due to the plants enhanced nutritional value (Jung et al., 2012). Due to new EU regulations and pathogens evolving resistance to current protection methods, there is increasing interest in sustainable alternatives to conventional crop chemicals (Hillocks, 2012). Consequently, AMF may also have scope to reduce pesticide applications to crops.

### **1.2.3 AMF in agricultural soils**

Most globally essential crop plants form associations with AMF. Therefore, the nutritional and non-nutritional benefits associated with AMF have led to increasing interest in how AMF might contribute to sustainable agriculture. AMF may have the potential to reduce the necessity for high nutrient inputs while maintaining high crop yields and may have additional benefits such as reduced

damage from plant pests and pathogens. Unfortunately, in agricultural soils AMF abundance and diversity has been reduced by over-application of nutrients, tillage regimes, long fallow periods and crop rotations, which disturb hyphal networks and create long periods where AMF have limited access to host plants (Helgason et al., 1998; Daniell et al., 2001; Jansa et al., 2002; Oehl et al., 2003).

Agricultural soils frequently show a reduction in AMF diversity, often dominated by *Glomus* species with reductions in genera such as *Gigaspora*, *Scutellospora* and *Acaulospora* (Helgason et al., 1998; Daniell et al., 2001; Jansa et al., 2002). Furthermore, damaging agricultural practices may have selected for AMF species with less plant-beneficial traits, such as higher investment in their reproduction over expending energy foraging for nutrients (Verbruggen & Kiers, 2010). For example, it is thought certain species of AMF can survive and maintain infectivity even when hyphal networks are disturbed due to high sporulation, meaning they are less dependent on an existing hyphal system to colonise host plants.

Due to the agricultural environment selecting for traits such as high sporulation over hyphal network extension and nutrient scavenging, it may be beneficial to increase the diversity and abundance of AMF populations in agricultural soils. AMF diversity, and abundance could be improved through changes to management practices which promote the abundance of native AMF or adding more AM fungi through inoculation. For example, a meta-analysis found AMF colonisation of crop plants was increased most when AMF inocula were applied to the soil (29% increase), followed by shortening the fallow period (20%) and then reducing soil disturbance through tillage (7%) (Lekberg & Koide, 2005). The significant increases in colonisation associated with these changes in management practices indicate that the inoculum potential of the soil is often limiting AMF colonisation. AMF inoculation and shortened fallow were also associated with increases in yield, biomass and shoot P concentrations ([P]), whereas reduced disturbance had no significant effects on yield or biomass despite increases in shoot [P].

#### **1.2.4 Commercially produced AMF inoculum**

Due to concerns about reduced spore densities and diversity of AMF in agricultural soil, there has been a growing number of organisations producing and selling mycorrhizal inoculants over the last decade (Vosátka et al., 2012; Faye et al., 2013). AMF inoculants are marketed to increase the abundance of

spores and the diversity of AMF species in agricultural soils and potentially enhance plant nutrient uptake and growth (Vosátka et al., 2012; Faye et al., 2013).

For AMF inoculants to move to the forefront of agriculture, they will need the ability to successfully compete with conventional chemical fertilisers in terms of cost and ease of application. Currently, the cost of AMF inocula is driven up through limitations of producing it on a large scale and through registration processes on microbial products which can be lengthy and expensive (Malusá et al., 2012). AMF are bio-trophic and therefore are difficult to culture as they need to be in association with a suitable plant host. However, recent progress has been made towards generating a pure culture of AMF (*Rhizophagus irregularis*); adding fatty acids (myristate) to the culture medium stimulated AMF growth and sporulation in the absence of a plant host (Sugiura et al., 2019).

An additional barrier to overcome in the production of inoculants is quality assurance, as the only way to guarantee pure, uncontaminated AMF inoculum is culturing fungi on transformed roots in axenic conditions (Vosátka et al., 2012). Currently, most inoculum production occurs in greenhouses with inert substrates and plants inoculated with pure fungal cultures. This method can produce up to 100 thousand spores per litre of the substrate, which is then diluted with a carrier before being sold (Malusá et al., 2012). However, contamination with other microbes is common (Vosátka et al., 2012), and studies have shown such inoculum is often contaminated by unwanted fungal species (Faye et al., 2013).

The choice of the carrier for AMF inoculum can have important implications of shelf life and protection of the microbes during transport and storage (Malusá et al., 2012), which is critical for the feasibility of inoculum use within the agriculture industry. In some studies, AMF inoculants have failed to produce any root colonisation in the target plant, and any growth increases seen from inoculants are attributed to nutrients within the substrate (Vosátka et al., 2012). Furthermore, molecular analysis using AMF-specific primers has failed to find evidence of species survival and persistence after inoculum addition (Berruti et al., 2016). The very low colonisation levels suggest viability may have been lost during shipping and storage (Corkidi et al., 2004; Wiseman et al., 2009), or AMF inoculum failed to compete with pre-established native AMF communities in the soil. There is also a risk that AMF species introduced as inoculum could fail to establish if they are

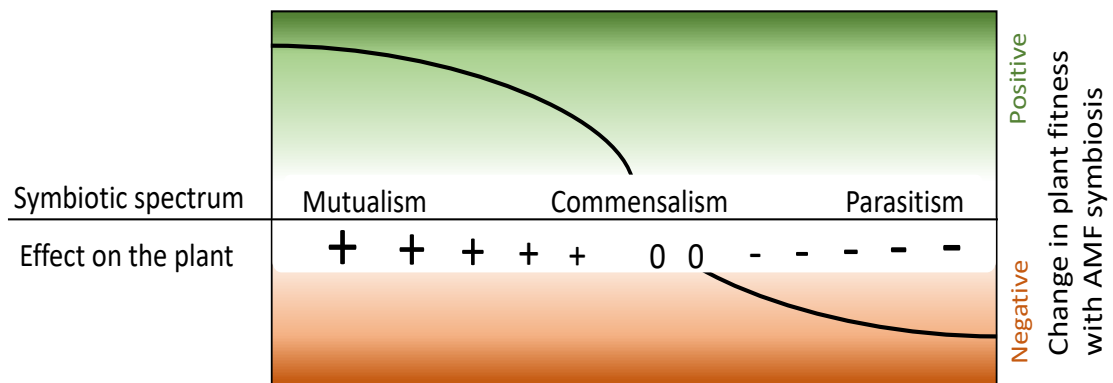
not compatible with the environment (Verbruggen et al., 2012). For example, differences in tolerance to disturbance (Schnoor et al., 2011), high nutrient levels in the soil (Zhao et al., 2017a) pH, soil type (Lekberg et al., 2007; Oehl et al., 2010), and heavy metal pollution (Hassan et al., 2011), have been found among AMF species (see section 1.2.5.3). In the future, quality control of commercial inocula will be imperative to its success, along with small scale tests of the inoculum's suitability to a certain plant host and environment before large scale field application is commenced, and AMF inoculum's establishment and the potential changes to the AMF community in the soil must be studied.

Another important consideration is that the inoculum comes in a form which is easy to apply and compatible with current agricultural practices (Malusá et al., 2012). Utilising machinery already available on farm could achieve this, for example adapting equipment currently used for spraying pesticides for the use of spraying AMF inoculum. Alternatively, AMF inocula could be applied through spraying over the soil surface with seeds, in-furrow application or as a seed coating (Adholeya et al., 2005).

Initial applications of AMF inoculants to restore community diversity and abundance may be costly; however, with the adoption of non-harmful management strategies such as reduced tillage and cover crops, repeat applications may not be necessary. Studies have demonstrated that AMF inoculants can be applied to agriculture in a commercially viable way and increase crop yields (Ceballos et al., 2013; Hijri, 2016). Hijri (2016) evaluated the effect of *R. irregularis* on potato yields in 231 field trials. The fields were maintained under conventional agricultural management, and application of inoculum was used at commercially viable levels using a liquid suspension of AMF spores applied to seeds via already available farm equipment. Inoculation was found to increase potato yields by an average of 9.5% and resulted in the application of AMF being not just commercially viable but profitable in most field trials. Importantly, they still found a reduction in yield in 14.6% of the field trials; this suggests AMF inoculants may not be advantageous in some situations and potential gains from AMF application may need to be assessed on a case-by-case basis.

### 1.2.5 What determines AMF inoculant outcomes?

It is well established that the symbiosis between AMF and a plant partner exists on a continuum, where the result for the plant host can be positive, neutral or negative (Figure 1.2) (Johnson et al., 1997; Klironomos, 2003; Hoeksema et al., 2010). Many biotic and abiotic factors determine, where the symbiosis lies on the continuum, including mycorrhizal species (or isolate) (Klironomos, 2003; Munkvold et al., 2004) host plant species (or cultivar) (Ellouze et al., 2016; Sawers et al., 2017; Watts-Williams et al., 2019) and levels of soil nutrients (Lekberg & Koide, 2005; Hoeksema et al., 2010). It is critical to understand the mechanism behind this variation in growth responses, so the decision of whether to use AMF inoculants can be accurately assessed on a case by case basis, and agricultural management systems can be designed that ultimately lead to an increase in crop biomass. Otherwise, AMF inoculants may become problematic in agricultural systems, where crop cultivars grown, and environmental conditions change frequently.



**Figure 1.2: The mutualism-parasitism continuum of mycorrhizal symbiosis concerning its effect on plant fitness.** There are three potential outcomes of the symbiosis, mutualism if the effect on plant fitness is positive, commensalism if the symbiosis has a neutral effect on the plant and parasitism if AMF associations reduce plant fitness.

#### 1.2.5.1 Plant-fungal specificity

Traditionally, the wide range of plant species which could form mycorrhizal associations compared to the low diversity of identified mycorrhizal species led

to the assumption that the symbiosis generally lacked strong host specificity, as presumably one fungal species must be able to form symbioses with many plant species (Smith & Read, 2008). Indeed, experiments have shown AMF isolated from particular plant species, will usually colonise any other plant species capable of forming AM associations (Smith & Read, 2008). Plants also need to be adaptable to the AMF in their environment, and any mutualist might be better than none at all. Alternatively, as environments change the most mutualistic fungal partner may also change, meaning maintaining flexibility is advantageous (Johnson et al., 1997), and AMF communities within plants have been shown to change over seasons and years (Dumbrell et al., 2011; Cotton et al., 2015).

However, it is clear symbiotic outcomes are largely variable, not only between species but also between genotypes or cultivars of plants and isolates of AMF (Tawaraya, 2003; Munkvold et al., 2004; Hoeksema et al., 2010). Additionally, testing multiple plant-fungal combinations has shown symbioses range from greatly mutualistic or parasitic depending on the plant and fungal combinations, with no single fungal species emerging as the best mutualist among all plants (Klironomos, 2003). Moreover, studies in natural systems have shown co-occurring plant species can have distinct AMF communities colonising their root systems (Helgason et al., 2002; Vandenkoornhuysen et al., 2002), and this naturally occurring specificity may be the most mutualistic (Helgason et al., 2002). This was the case with *Acer pseudoplatanus* which naturally associated almost exclusively with *Glomus hoi* (now *Simiglomus hoi*), and when experimentally combined with other AMF species only *G. hoi* enhanced P acquisition (Helgason et al., 2002). Plant-fungal specificity could be an important consideration when applying commercial inocula in agricultural soils, and some AMF species may be better at enhancing nutrient assimilation in certain crops or cultivars than other AMF species.

*Rhizophagus irregularis* (previously, *Glomus intraradices*) is emerging as an AMF strain with highly flexible application potential to a wide array of environments and crops (Ceballos et al., 2013; Hijri, 2016; Köhl et al., 2016). *R. irregularis* is the model species of AMF and the first to have its genome published. However, even within the reference strain DAOM197198, 32 variations were found on the ribosomal internal transcribed spacer (ITS) regions of cultures propagated in different laboratories (Stockinger et al., 2009), demonstrating the high genetic



variability across AMF species. One study compared the gene content of model *R. irregularis* with five other sequenced isolates and found a striking number of isolate-specific genes, indicating diversity within isolates which is ten times higher than within distant fungal relatives (Chen et al., 2018). This high genetic variability between the isolates of *R. irregularis* can produce substantially different host biomass and nutrient uptake responses (Angelard et al., 2010; Mensah et al., 2015; Koch et al., 2017). However, the underlying mechanism behind variation in symbiotic outcomes due to plant-fungal specificity is still largely unknown, representing a significant barrier to the wide-scale uptake of AMF in agriculture, without case-by-case assessment before of the inoculum's suitability to a certain plant host.

### **1.2.5.2 Competition**

One critical question relevant to the success of agricultural inoculation with AMF is whether commercial inocula will be able to compete with the native community to form a successful association with the targeted host plants and increase crop yields. The outcome of competitive interactions between the added AMF inoculant and the indigenous community may depend on multiple factors, for example, whether the species within the inoculum are compatible with the target environment (see section 1.2.5.3).

Alternatively, the establishment of added AMF may depend on the extent to which a particular system can support an increasing AMF population, defined as the carrying capacity of the soil and/or the plant hosts. The carrying capacity is strongly related to the presence of a suitable host plant which provides C to the AMF. For example, some agricultural systems may have a low carrying capacity due to fallow periods and crop rotations with non-host plants. Alternatively, high P levels in the soil often reduce root colonisation within the host plant, and therefore, AMF population growth (Verbruggen et al., 2012).

Consequently, if the native AMF community's abundance in an agricultural environment is at carrying capacity, the addition of a commercial AMF inoculum will be unlikely to increase the abundance of AMF and root colonisation without changes in management practices to increase carrying capacity. Competition between indigenous and introduced species at the limit of soil carrying capacity could result in adverse effects for the host plant (Janoušková et al., 2013). The negative effects of competition are theoretically due to AMF allocating resources

to competition for space and C, for example, increases in the intraradical mycelium (IRM) and vesicles structures, rather than investing in structures for nutrient uptake and plant benefits such as arbuscules and extraradical mycelium (ERM).

However, results of meta-analyses indicate that plant productivity in agricultural soil is often limited by low inoculum potential (the amount of viable and infective fungal propagules within the soil) (Lekberg & Koide, 2005). It is possible that adding AMF inoculum to soils with already present AMF communities can cause a significant growth response in plants, with this response decreasing as the natural community abundance increases, suggesting agricultural soils are not typically at carrying capacity (Lekberg & Koide, 2005).

It has been hypothesised that more closely related fungal species will compete against each other more vigorously (Maherali & Klironomos, 2007). It is hypothesised that species assemblages are determined by competitive interactions that will limit the long-term co-existence of species with similar fundamental niches, this exclusion will lead to more phylogenetically diverse species co-existing (Maherali & Klironomos, 2007). This hypothesis was tested in a plant-AMF symbiosis model using *Plantago lanceolata* and experimentally constructed AMF communities which differed in their phylogenetic relatedness. After one year, species richness was highest in the phylogenetically diverse communities, which retained >80% of initial species richness. In contrast, communities constructed with species mainly in the same family preserved <40% of their original richness. More distantly related species may reduce competitive pressure by colonising distinct zones within the root (Maherali & Klironomos, 2007). Alternatively, phylogenetically diverse species may be functionally different and complementary, which could benefit the plant host due to different abilities to acquire nutrients or provide non-nutritional benefits such as defence. However, how the plant could promote this diversity mechanistically is unknown, illustrating the little-understood complexity of host plant interactions with AMF communities.

If competition between AMF species is driven by phylogenetic relatedness, then commercial inocula may be most successful at increasing AMF diversity when not in competition with similar species. However, fungal communities in the field are likely to be strongly influenced by abiotic factors, which may even surpass the

influence of phylogenetic effects (Maherali & Klironomos, 2012). A global meta-analysis of AM fungal community structure found that community composition at a site is more likely to be phylogenetically clustered (Kivlin et al., 2011), suggesting species composition on a larger scale is restricted by an ecological filter associated with niche requirements and environment.

### **1.2.5.3 Abiotic factors**

Environmental factors must be considered when AMF species are introduced into a new environment (Verbruggen et al., 2012). For example, native species are likely to be better adapted to local conditions and this will be particularly important in extreme environmental conditions. In land with high salinity (Estrada et al., 2013), or high levels of heavy metals (Briccoli Bati et al., 2015) for instance, native species have been found to be more tolerant and successful at increasing the tolerance of their host plants to harsh conditions. High salinity severely limits the germination and hyphal extension of some AMF species (Juniper & Abbott, 2004). Therefore, for specialist situations such as soil with high salinity or heavy metal contents, tolerance to the environment should be considered when designing AMF inocula.

Many recent studies have found that soil properties play an essential role in determining AMF community structure (Lekberg et al., 2007; Dumbrell et al., 2010a; Verbruggen et al., 2012; Moebius-Clune et al., 2013; Jansa et al., 2014), with various lines of evidence suggesting different AMF species are dominant in different soil types, and this may have a stronger effect on community structure than agricultural management practise (Jansa et al., 2014). However, it has proved problematic to identify the mechanism causing this effect as many correlated factors have been implicated such as texture, pH, soil organic C content, nutrient concentrations and level of moisture (Lekberg et al., 2007; Moebius-Clune et al., 2013).

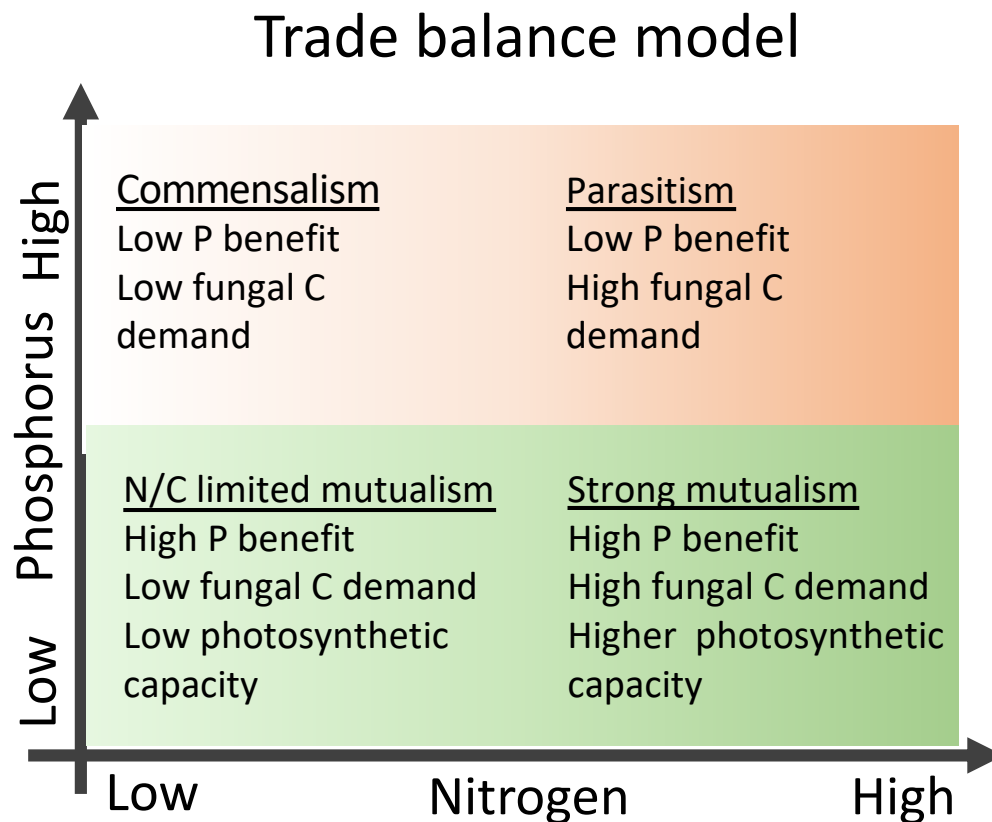
Sand and clay content have been shown to have a niche partitioning effect on different AMF families (Lekberg et al., 2007). For example, *Gigasporaceae* species are often found to be more successful in soil with high sand content whereas *Glomeraceae* species are more dominant in soil with high clay content (Mathimaran et al., 2005; Jansa et al., 2014). The mechanism behind this effect is unclear; there is some evidence that the family's alternative growth strategies could be affecting their success rates in each soil type. *Gigasporaceae* and

*Glomeraceae* have contrasting patterns of hyphal growth (Maherali & Klironomos, 2007; Smith & Read, 2008), with *Gigasporaceae* producing extensive external hyphae. Close textured heavy soils such as those with high clay content may be obstructive to hyphal extension causing problems for *Gigasporaceae*. *Gigasporaceae* abundance is reduced in soil with high clay content regardless of any competition from other fungal species.

In an agricultural environment, it is also vital to consider tolerance to disturbance when designing AMF inocula. Land management intensity influences AMF diversity and community structure, and tillage has the most drastic effect on AMF communities within agricultural systems (Helgason et al., 1998; Daniell et al., 2001; Jansa et al., 2002; Oehl et al., 2010). The high nutrient contents maintained within agricultural soils are a further abiotic factor which could limit the success of commercial inocula. Concerns exist that AMF inocula won't be able to provide a substantial benefit through additional nutrient acquisition as soil nutrient concentrations (particularly P) increase, and therefore the growth benefits attained through AMF will decline (Smith & Read, 2008). Although AMF may still contribute substantially to plant P uptake, growth will likely be limited by other factors such as N or the rate of C acquisition. Certainly, low irradiance in experiments is often a trigger for negative growth responses to AMF (Son & Smith, 1988; Zhu et al., 2001), suggesting the costs incurred by supporting a biotrophic fungal partner were higher than the benefits of nutrient acquisition from the symbiosis.

However, results on the success of AMF inocula at high nutrient concentrations have been conflicting (Johnson et al., 2010; Antunes et al., 2012; Cozzolino et al., 2013; Köhl et al., 2016), and may be explained when the comparative availability of C, N and P are considered together in the 'Trade-Balance Model' (Johnson et al., 2010) (Figure 1.3). N supply is strongly linked to both photosynthesis and fungal C demand; therefore, in P-rich soils, where the symbiosis has little benefit to the host, N supply can be detrimental to the mutualism by increasing the AMF C sink strength. Therefore, in high input systems, the use of commercial inocula should be evaluated carefully as C costs may outweigh the benefits provided through nutrient returns. Future agriculture should aim to reduce nutrient inputs in a way to provide maximum benefits from the symbiosis, for example, the addition of AMF inoculum as a partial substitution

for high P fertiliser applications has produced comparable yields to conventional fertiliser treatments (Cozzolino et al., 2013).



**Figure 1.3: Trade balance model scenarios, showing hypothesised plant benefits depending on the relative availability of nutrients in the soil.** Nitrogen (N) availability can influence both carbon (C) gained by the plant through photosynthesis and also the C demand by the size of the fungal network, whereas phosphorus (P) regulates the main nutrient benefits gained from the association (modified from Johnson, 2010).

On the other hand, increases in plant C supply and ‘excess’ photosynthate to support the symbiosis may reduce the chance of negative growth responses if C transfer to the fungal partner is high. How the symbiosis will change as atmospheric C rises, further evolving the resource availability dynamics within the mutualism, is still mostly unknown.

### 1.3 The impact of rising atmospheric [CO<sub>2</sub>] on AM symbiosis

Adaptation to climate change involves exploring the potential advantageous opportunities associated with climate change, for example, longer growing

seasons or increased yields associated with rising [CO<sub>2</sub>] (see section 1.1.2). Another one of these opportunities which should be investigated is the potential for a stronger mutualism between AMF and C3 crop plants such as wheat.

Rising [CO<sub>2</sub>] has been shown to have a drastic effect on the efficiency of photosynthesis and consequently the growth of many of our crop species, namely C3 plants such as wheat (Mitchell et al., 1993). Elevated [CO<sub>2</sub>] could mitigate any adverse effects of AMF on plant growth, which are associated with a drain on the plant's C resources. Negative plant growth responses to AMF found at high P fertilisation levels under ambient [CO<sub>2</sub>] were mitigated at elevated [CO<sub>2</sub>] in *Brachypodium distachyon*, suggesting any C "drain" on the plant elicited by the AMF was compensated by increased C assimilation via enhanced photosynthetic rate at elevated [CO<sub>2</sub>] (Jakobsen et al., 2016). As plant C fixation increases, C is less likely to be the plant's limiting resource, and this could also mitigate any C limitation in AMF development and stimulate formation of AM associations.

AMF may, in turn, fulfil the plant's increased need for essential nutrients such as N and P at elevated [CO<sub>2</sub>] to enable maximum growth responses. This is particularly important as evidence suggests faster growth rates brought upon by elevated [CO<sub>2</sub>] can result in lower protein contents in the cereal grains, due to shortened periods of nutrient acquisition (Myers et al., 2014). Decreases in the nutritional value of cereal crops where they are staple foods jeopardies the nutrition-related health of millions of people. Plants have been shown to invest higher amounts of C below ground as [CO<sub>2</sub>] increases (Drigo et al., 2010, 2013; Field et al., 2012, 2015a), suggesting a possible rise in C supply to AMF symbionts which has been shown to increase mycorrhizal biomass (Drigo et al., 2007). It is essential to elucidate whether this could increase nutrient uptake of the plants in return. The effect of changing [CO<sub>2</sub>] on crop-mycorrhizal symbioses remains little investigated. Due to the increasing interest in the application of commercially available AMF inoculants in agriculture, how fungal inoculants will respond to future climates represents a critical knowledge gap and a potential barrier to future sustainability in agriculture.

## 1.4 Aims and Hypotheses

The overarching objective of this thesis is to determine the effect of application of a commercial AMF inoculum (the generalist species *R. irregularis*) on crop growth and nutrition in a changing climate, and thereby the ability of AMF inoculation of food crops to contribute to plant nutrient uptake now, and in the future. The following key questions and hypotheses are addressed:

### Key Questions

1. What is the effect of *R. irregularis* inoculation of agricultural soil on wheat biomass and carbon-for-nutrient exchange within the AM symbiosis?
  - Applying a *R. irregularis* inoculum to agricultural field soil could result in greater root colonisation and increased fungal-acquired nutrient assimilation by wheat plants. Inoculated plants are expected to allocate more plant-fixed C to their AMF symbionts; thereby nutrient gains will be offset by a greater C cost to the plants.
2. Does inoculation with *R. irregularis* cause changes to the wheat root fungal community assembly?
  - Addition of a *R. irregularis* inoculum will result in changes to the mycorrhizal community found within the plant root system with increased dominance of *R. irregularis* and a reduced native AMF diversity and richness.
3. How will increasing atmospheric CO<sub>2</sub> concentrations affect carbon-for-nutrient exchange between arbuscular mycorrhizal fungi and wheat?
  - Wheat, a C<sub>3</sub> crop, will have improved C fixation at elevated [CO<sub>2</sub>], and this will drive greater C allocation to fungal symbionts and increased fungal biomass. Increased AMF biomass will result in the increased nutrient acquisition of the plant through its fungal partner. Therefore, use of commercial AMF inocula will be more advantageous for plant nutrient gain and growth increases in a future climate scenario.

## **Chapter 2 : The effect of inoculation with *Rhizophagus irregularis* in agricultural soil on wheat yield and carbon-for-nutrient exchange within the AM symbiosis.**

### **2.1 Introduction**

Improving the efficiency of food production and closing yield gaps are essential elements of ensuring food security in the future (IPCC, 2018). Grain harvests have doubled over the past fifty years. Increases in food production have resulted from the development of high yielding cultivars, chemical fertilisers, pesticides and irrigation strategies developed since the Green Revolution of agricultural innovation. The invention of new insecticides, herbicides and fungicides from the 1960-80s significantly improved pest control (Aktar et al., 2009), and the use of irrigated cropland and synthetic fertilisers all contributed greatly to agricultural output (Foley, 2005). However, crop yields have now plateaued (Grassini et al., 2013), and continued use of large amounts of nutrient fertilisers is unsustainable.

Phosphorus-based fertilisers are derived from mining rock phosphate, which is a non-renewable resource. It is estimated that global reserves of rock phosphate will be exhausted within the next 300 years (Cordell et al., 2009; Van Vuuren et al., 2010; Cordell & White, 2013). Furthermore, a large proportion of the greenhouse gases (GHG) created during food production are a result of manufacturing ammonium-nitrate based fertilisers. For example, over 40% of the GHG emissions produced making a loaf of bread, come from the production of ammonium-nitrate fertilisers alone (Goucher et al., 2017). Ammonium-nitrate fertilisers produce triple the emissions of the rest of the cultivation processes combined, which come to 14%, and also more than baking and transport which account for 29% and 9% respectively. Overuse of both nitrogen and phosphorus-based fertilisers results in substantial environmental pollution through run-off and eutrophication (Robertson & Vitousek, 2009; Cordell & White, 2013).

To reduce dependence on fertilisers, N and P resources already present in the soil need to be utilised more effectively while maintaining or improving plant growth and nutrient uptake to ensure food security. Plants can acquire nutrients through two pathways (Smith et al., 2003). The first is the direct pathway, which consists of direct absorption of nutrient through the root cells. The second is the



mycorrhizal pathway, whereby plants get nutrients through forming associations with mycorrhizal fungi (see section 1.2.1).

Given the capacity for arbuscular mycorrhizal fungi (AMF) to increase host plant nutrient assimilation (see section 1.2.1), there is potential to exploit the symbiosis within an agricultural context to reduce P and N fertiliser inputs (Sangabriel-Conde et al., 2014; Zhu et al., 2016; Püschel et al., 2017). However, intensive crop production has been shown to reduce AMF spore densities and diversity in the soil and subsequently result in low rates of colonisation of plant roots (Helgason et al., 1998; Daniell et al., 2001; Jansa et al., 2002; Oehl et al., 2003; Bowles et al., 2017).

The instability and heterogeneity of the agricultural soil environment, with high levels of disturbance and nutrient fluctuations, may result in selection for fungal species whose life history strategies are suited to unstable environments, for example, short lifecycles and high reproductive outputs (Verbruggen & Kiers, 2010; Chagnon et al., 2012). In agriculture, AMF species with these weedy traits may replace species that are potentially more mutualistic (i.e. species that confer greater nutritional benefit on their host plant), with characteristics adapted for nutrient acquisition, such as investment in extra-radical hyphae (Verbruggen & Kiers, 2010).

The abundance and diversity of AMF in the soil could be improved by changes to management practices to promote increases in native AMF within the soil or by directly adding more AMF through commercially available inoculants (Vosátka et al., 2012; Faye et al., 2013). Many commercially available AMF inoculants are marketed to be able to increase plant nutrient uptake and growth, however, the success of AMF inoculants has been highly variable (Vosátka et al., 2012; Faye et al., 2013). In some cases this could be due to a complete failure of AMF species within the inoculum to establish in the new environment (Vosátka et al., 2012). Lack of AMF establishment may be due to low viability within the inoculum itself, incompatibility with the target environment or inability to compete with pre-established AMF species in the environment (Verbruggen et al., 2012) (see section 1.2.5).

However, even when inoculum establishment is successful at increasing the colonisation of host plant roots, this does not always correspond to increases in plant growth and nutrient uptake (Pellegrino & Bedini, 2014; Köhl et al., 2016;

Zhang et al., 2019). Whether the outcome for the plant host is positive neutral or negative can depend on many factors including the species or isolate of AMF partner (Klironomos, 2003; Munkvold et al., 2004), and many abiotic factors such as soil nutrient levels (Lekberg & Koide, 2005; Hoeksema et al., 2010) (see section 1.2.6) (Figure 1.2). It is also possible that non-native AMF introduced to an environment through the use of AMF inoculants, may be less compatible with native plant species and be more likely to produce neutral or negative growth responses (Helgason et al., 2002; Klironomos, 2003; Pellegrino et al., 2011; Řezáčová et al., 2017).

In order to evaluate whether AMF inoculants could be a valuable part of a sustainable management strategy in agriculture, including being economically viable for farmers, it is critical to understand what determines whether an AMF inoculant will be successful at increasing plant growth and nutrient uptake. The decision of whether to use AMF inoculants for specific crops in agriculture can then be accurately assessed on a case by case basis. If AMF inoculants are implemented without an adequate understanding of what causes negative plant growth responses, they may become problematic in agricultural systems, where crop cultivars grown, and environmental conditions change frequently.

There is a long-held view that cereal crops such as wheat do not respond positively to AMF associations, even when receiving nutrients through the symbiosis (Hetrick et al., 1993; Zhu et al., 2001; Ryan et al., 2005; Li et al., 2006). Wheat may be unresponsive to AMF symbiosis due to commercial breeding programmes focusing on selection for root systems that are highly effective at assimilating necessary nutrients from mineral fertilisers (Tawaraya, 2003), leading to development of modern crop cultivars (after 1950) that are less reliant on AMF (Hetrick et al., 1993; Zhu et al., 2001; Zhang et al., 2019). Additionally, there is limited evidence that breeding for resistance to fungal diseases may impair AMF colonisation of crop plants, for example when disease-resistant lines of maize were compared with susceptible lines, AMF colonisation was reduced within disease-resistant lines (Toth et al., 1990).

On the other hand, variation may be due to cultivar specific responses to inoculation with AMF, which have been shown many times in wheat (Ocampo & Azcon, 1981; Hetrick et al., 1993; Zhu et al., 2001). In thirteen wheat cultivars inoculated with *Glomus mosseae* (now *Funneliformis mosseae*), a range of

positive to neutral growth responses was observed, which were not related to colonisation. The only explanatory factor found was root mass, with the cultivars with the smallest root systems benefiting most from AMF inoculation. Plants with large root systems may not respond positively to AMF due to their ability to absorb adequate P without an AMF partner. However, root mass is unlikely to be the only factor, and cultivar-specific responses to *G. mosseae* should be considered. In maize, increases in shoot biomass and P uptake when inoculated with *R. irregularis* also varied between cultivars (Chu et al., 2013).

A recent study looking at plant and fungal gene transcription during the symbiosis between five cassava cultivars and two genotypes of *R. irregularis* found that 72% of genes affected by the symbiosis were transcribed differently in either direction or extent dependent on cassava cultivar (Mateus et al., 2019). Substantial differences in transcription were also found between fungal isolates, suggesting a plant genotype/fungal genotype interaction. However, the underlying cause behind variation in cultivar responses to AMF is still mostly unknown, representing a substantial barrier to the wide-scale uptake of AMF in agriculture.

Studies indicating the unpredictable responses of some of our modern crop cultivars to AMF, and a lack of knowledge on what determines mycorrhizal growth responses of different crop genotypes, led to a review which suggested farmers should not consider the management of AMF when trying to improve crop nutrient uptake and yield (Ryan & Graham, 2018). However, this is challenged in a recent meta-analysis which found AMF inoculation in the field led to a 16% increase in grain yield assessed over seven cereal crops, in wheat, the increase was 17% (Zhang et al., 2019). However, the study still notes variation with only 77% of trials having an increase in grain yield; this is in agreement with previous meta-analyses (Lekberg & Koide, 2005; Pellegrino et al., 2015).

The causes of variation and particularly any negative growth responses that occur in plants when associated with AMF remain unknown. It is possible that large amounts of plant photosynthetically derived C are reallocated to the fungal partner. Studies have suggested that different species of AMF vary in the C costs they generate for their host (Lendenmann et al., 2011). However, in *Gigaspora* the observed high C costs to *Medicago* and low P uptake were not accompanied by the expected negative growth responses, as seen in other studies (e.g. in

wheat (Li et al., 2008)). A concurrent increase in C fixation explained the lack of a negative growth response. Previous studies have also suggested AMF plants upregulate photosynthetic rates, which compensates for any C loss to the fungal partner (Wright et al., 1998; Kaschuk et al., 2009). However, evidence suggests this is not universally true, and there are still numerous reports linking negative plant growth responses to AMF to a significant C drain (Graham & Eissenstat, 1998; Jones & Smith, 2004).

Alternatively, it could be that association with AMF causes a substantial reduction in the direct pathway for nutrient uptake within the plant and the AMF pathway is unable to compensate (Figure 1.1) (Li et al., 2008; Smith et al., 2009; Smith & Smith, 2011). It has recently been accepted that P uptake from the direct and AMF pathways are not additive (Li et al., 2008; Smith et al., 2009; Smith & Smith, 2011). This means a mycorrhizal contribution to P cannot be measured through assessing differences in P between mycorrhizal and non-mycorrhizal plants and AMF can make a considerable contribution to plant P while not affecting overall [P].

It is crucial to determine whether C drain or reduction in the direct-uptake pathway can be linked to negative growth responses when considering the use of AMF inoculants in agriculture. AMF inoculant additions are likely to result in larger AMF populations dependent on the plant host for C resources, which then could exacerbate negative growth responses due to increased C drain. Stable and radioactive isotope technology allows tracking of C, P, and N through AMF-plant symbiosis (Lekberg et al., 2010; Lendenmann et al., 2011; Schnoor et al., 2011; Fellbaum et al., 2012), and could help establish what determines the outcome of the symbiosis as either, positive, neutral or negative.

Within this experiment, I determined whether the addition of a commercially-available (*Rhizophagus irregularis*) inoculant to a non-sterile agricultural soil could contribute to enhancing nutrient uptake in wheat. Cereal crops, including wheat, are highly unlikely to remain uncolonised by AMF during field cultivation, where communities of AMF are present in the soil (Helgason et al., 1998). Therefore, to assess the realistic benefits of application of AMF inoculum in agriculture, it is essential to investigate the potential benefits of inoculation using non-sterile soil from an agricultural field where a native AMF community is present. I used this rationale in my experiments to investigate the ability of a

commercial available inoculant to increase wheat root colonisation, plant P and N uptake, biomass and yield.

I measured whether the inoculant could produce consistent results across three wheat cultivars, due to evidence that response to AMF can vary considerably across crop cultivars. The chosen cultivars are a mix of old (cv. Avalon and Cadenza) and new varieties (cv. Skyfall). The cultivars, especially cv. Avalon and Cadenza, have differences in canopy architecture, root system size and disease resistance, which may contribute to differences in response to AMF inoculation.

## 2.2 Aims and hypotheses

The overarching aim of this study was to quantify the ability of mycorrhizal fungi (*Rhizophagus irregularis*) introduced via application of a commercially-available inoculum to contribute to improved plant growth and nutrient uptake balanced against the cost of the symbiosis. I measured responses to inoculation with *R. irregularis* in three wheat cultivars to assess the extent of variability between cultivars.

I investigated whether the addition of a commercially available AMF inoculant (*Rhizophagus irregularis*) provides any benefit to wheat in terms of nutrient uptake, growth and yield. I used  $^{14}\text{C}$ ,  $^{33}\text{P}$  and  $^{15}\text{N}$  isotopes, to assess what contributes to the outcome of the symbiosis in terms of plant nutrient uptake and growth and if differences in C drain or nutrient uptake through the AMF pathway contribute to cultivar specific responses to AMF.

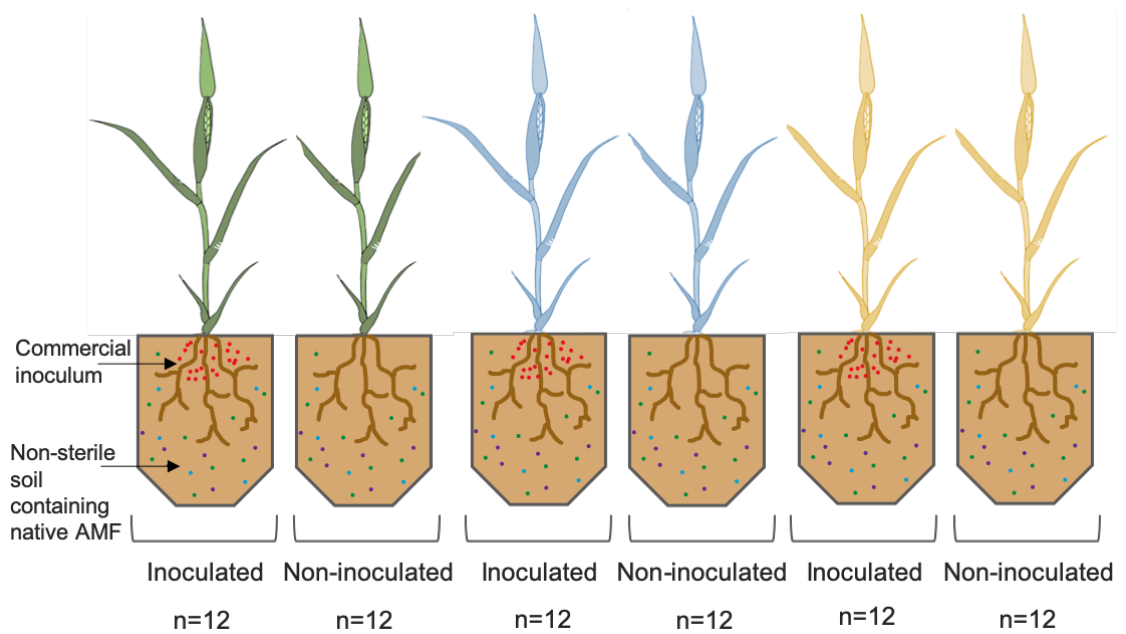
I test the following hypotheses:

1. *Rhizophagus irregularis* inoculum can increase plant root colonisation by AMF due to the low densities of native AMF spores and propagules in the soil, caused by damaging agricultural practices, being substantially enhanced by inoculation.
2. Wheat will have genotype-specific responses, varying according to cultivar, to inoculum additions. Cultivars with high resistance to fungal pathogens and larger root systems, such as cv. Cadenza may be less responsive to AMF. Cultivar-specific responses may manifest as differences in mycorrhizal fungal-acquired nutrient uptake, C allocated to the fungal partner, plant growth or yield.
3. Supporting a larger abundance of AMF within the plant root system, measured via root colonisation and soil-hyphal lengths, will result in a more substantial C drain on wheat hosts due to the obligate biotrophic nature of the AMF.

## 2.3 Materials and Methods

### 2.3.1 Biological material and growth conditions

Wheat seeds (*Triticum aestivum* L. – cultivars: Skyfall, Avalon and Cadenza) were sterilised with chlorine gas and subsequently germinated in Petri dishes on damp filter paper at 20°C for six days. Seedlings were then transplanted into an agricultural soil and sand substrate mix (1:1) in plant pots (1100 cm<sup>3</sup>). Agricultural soil was collected from Leeds university farm in February 2018 (Wise Warren Farm, Warren Lane, Tadcaster, North Yorkshire, LS24 9NU, England). The soil, a slightly alkaline sandy clay loam, was air dried over-night in trays and passed through a 2 mm sieve to ensure the substrate was consistent. The soil was then stored at room temperature for ten days before planting. Analysis of the soil characteristics showed soil organic C content represented ~2% of soil dry weight and soil solute concentrations of PO<sub>4</sub>, NO<sub>3</sub>, and NH<sub>4</sub> were 0.08 mg L<sup>-1</sup>, 6 mg L<sup>-1</sup>, and 0.04 mg L<sup>-1</sup> respectively (Holden et al., 2019). The soil used within this experiment was not sterilised, therefore all plants were exposed to the native AMF community within the agricultural soil. The seedlings were then further inoculated with either 20 g of a commercially available AMF inoculum containing a single species *R. irregularis* (PlantWorks Limited, Kent) or an autoclaved control inoculum i.e. no additional AMF, just the native AMF community in the agricultural soil (see Figure 2.1). The inoculum is produced in vivo using host plants clover and maize. The growing substrate is a mixture of pumice and zeolite (1:1 blend). The inoculum contains a mix of infective propagules including colonised root fragments, hyphae and AMF spores.



**Figure 2.1: Experiment treatments.** Including three wheat cultivars (indicated by the different coloured wheat plants), all grown in non-sterile agricultural soil with the native AMF community present. Half the plants within each cultivar (n=12), were then inoculated with a commercial AMF inoculum containing *Rhizophagus irregularis*.

The inoculum was placed in the potting hole at the time of planting (n=12 per experimental treatment: cultivar combination) (see further detail below). Greenhouse growing conditions were supplemented with LED lighting and electronic blinds to create a 16-hour photoperiod, light intensity:  $350 \mu\text{mol.m}^{-2} \text{s}^{-1}$ , average temperature:  $23^\circ\text{C}$ , the plants were watered every three days with each being given 30 ml of 40% nitrate type Long Ashton solution weekly (Hewitt, 1966).



**Table 2.1:** Stock solutions needed to obtain 40% Long Astons solution (nitrate type)

Compound	M.Wt	g. in 1L stock	Molarity of stock	ml/L for 40%	Dilution rate	Concentration of 40% (mM)
KNO <sub>3</sub>	101.1	50.6	0.5	3.2	312.5	1.60
Ca(NO <sub>3</sub> ) <sub>2</sub> anh	164.2	80.3	0.5	3.2	312.5	1.60
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	156.0	52.0	0.3	1.6	625	0.48
MgSO <sub>4</sub> .7H <sub>2</sub> O	246.5	46.0	0.2	3.2	312.5	0.64
FeNaEDTA	367.1	6.7	0.018	2	500	0.036
Micronutrients		*		0.4		

\* Micronutrient stock solution contains the following (g) in 1 litre:

MnSO <sub>4</sub> .4H <sub>2</sub> O	2.23
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.29
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.25
H <sub>3</sub> BO <sub>3</sub>	3.10
NaMoO <sub>4</sub> .2H <sub>2</sub> O	0.12
NaCl	5.85

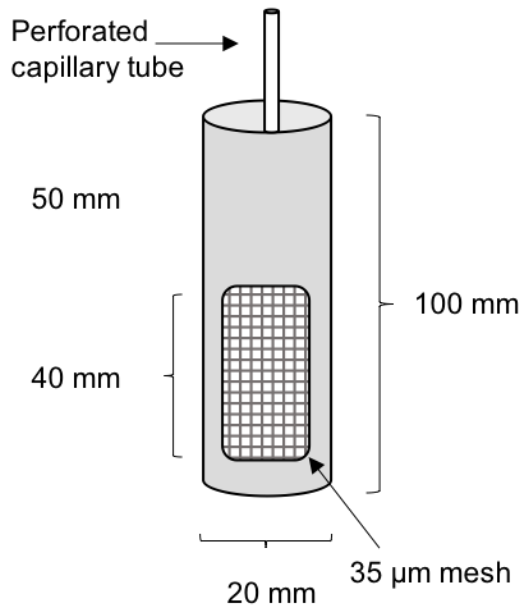
### 2.3.2 Measuring the efficiency of wheat-AMF symbiosis through isotope tracing experiments (<sup>33</sup>P, <sup>15</sup>N & <sup>14</sup>C)

#### 2.3.2.1 Experimental set-up

The wheat plants were grown for eight weeks in plant pots with an 1100 cm<sup>3</sup> soil volume, as described above (n=12 per experimental treatment). cv. Skyfall and Cadenza did not require vernalisation they were also grown to yield, under the same conditions as described above.

Two plastic cores, of 20 mm diameter and 100 mm long with 35 µm pore nylon mesh windows, were inserted into each pot (Figure 2.2). The cores contain a 1-mm diameter perforated capillary tube in the centre, fixed using a waterproof sealant (Aqua Mate, Ever Build, Dublin) applied to the end of the tube before it was placed onto the mesh at the bottom of the core. A third core was filled with glass wool and sealed using an air-tight rubber septum (SubaSeal, Sigma) (Figure 2.3). This core enabled below-ground gas sampling throughout the <sup>14</sup>C labelling period.

Plants were harvested immediately after completion of  $^{33}\text{P}$ - and  $^{15}\text{N}$ -for- $^{14}\text{C}$  tracing (Zadok growth stage GS30-39), this stage was chosen for isotope tracing as it represented a period of rapid growth and high nutrient demand where the crops may rely more heavily on AM-mediated nutrient uptake .

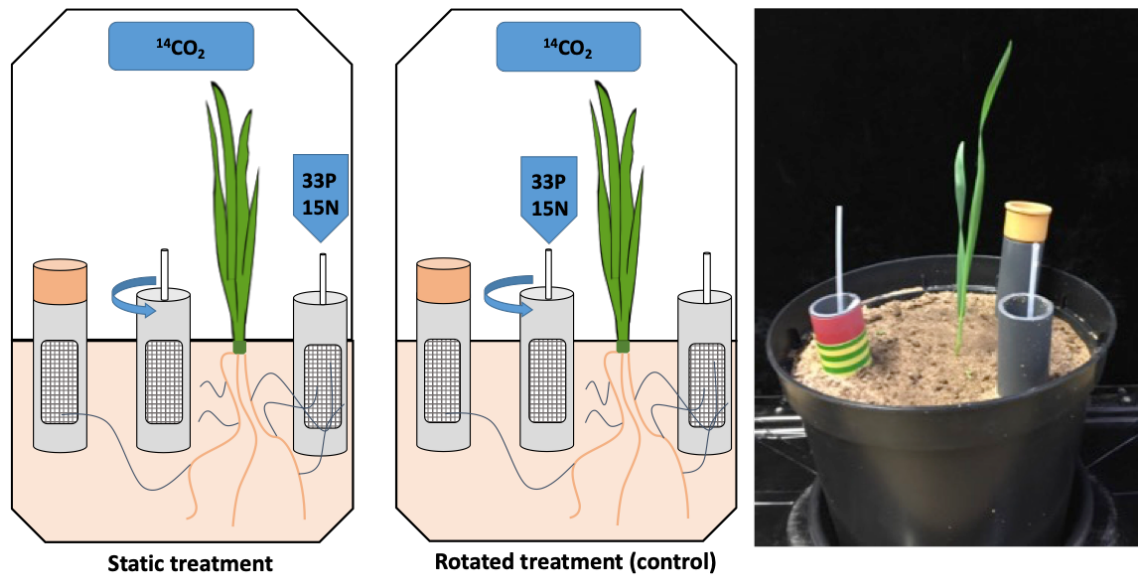


**Figure 2.2: Schematic diagram of mesh-covered core (not drawn to scale).**

### **2.3.2.2 Quantifying $^{33}\text{P}$ and $^{15}\text{N}$ for $^{14}\text{C}$ exchange.**

Forty five days after planting when the plants were between 6 and 7 weeks old, 150 µl of  $^{33}\text{P}$ -Phosphoric acid (1MBq; Hartmann Analytic, Germany) (Specific activity: cv. Skyfall – 174.9 TBq mmol<sup>-1</sup>, Cadenza -179.4 TBq mmol<sup>-1</sup>, Avalon – 180.6 TBq mmol<sup>-1</sup>) and  $^{15}\text{N}$  ammonium chloride (1.5mg/ml) (MP Biomedicals, Santa Ana, USA) was supplied to a mesh core through the perforated capillary tube. Diffusion of  $^{33}\text{P}$  and  $^{15}\text{N}$  out of the cores and other microbial nutrient cycling (such as decomposition of organic matter and recycling of available soil nutrients, which could be moved outside the core within bacteria or fungi) was controlled for by rotating half of the labelled cores within a treatment (Figure 2.3). Rotating the cores severed AM fungal hyphae connections between the isotopes and the plant, allowing the rate of diffusion and microbial nutrient cycling to be established by subtracting the rotated core and static core treatments. At the 8-week time point, plants were placed in sealed airtight chambers using large ziplock bags. Soil cores were sealed with scintillation vial caps and anhydrous lanolin. Shoots were supplied with  $^{14}\text{CO}_2$  within a sealed system for a 16-h photoperiod via the

liberation of 110  $\mu\text{l}$  Sodium bicarbonate [ $^{14}\text{C}$ ] 1 MBq (specific activity: all cultivars - 2.13 GBq  $\text{mmol}^{-1}$ ; Hartmann Analytic, Germany) using 2 ml of 10% lactic acid. At the end of the labelling period, 4 ml 2M KOH was injected into the sealed systems to trap remaining  $^{14}\text{C}$ .



**Figure 2.3 Experimental systems used for quantifying  $^{33}\text{P}$  and  $^{15}\text{N}$  for  $^{14}\text{C}$  exchange between mycorrhiza and plant partners.** The experimental system was labelled with  $^{33}\text{P}$ ,  $^{15}\text{N}$  and  $^{14}\text{CO}_2$ . In 50% of replicates, the labelled core was rotated to sever hyphal connections and control for isotope diffusion outside of the core. The third core with an airtight seal (orange) was used to track plant-fixed C passed to the fungal partner through fungal respiration of  $^{14}\text{C}$

### 2.3.2.3 Plant harvest and tissue analysis

The cores were removed from the pots, the substrate within the cores was collected along with all the plant and soil material and freeze-dried and weighed in the following parts: shoot tissues, root tissues, bulk soil, static core soil, rotated core soil. The substrate was carefully washed from the roots system using water. The fresh mass of the roots was recorded for each plant harvested, and a subsample of the roots was stored in 50% ethanol at  $4^\circ\text{C}$  until colonisation analysis. The total mass of root tissue could be quantified using the proportion of fresh mass to dry mass of the dried subsample. 1 ml of 2M KOH used to trap the remaining  $^{14}\text{C}$  was transferred to a scintillation vial with the liquid scintillant 10 ml Ultima Gold (Perkin-Elmer), and the radioactivity detected via scintillation counting (Packard Tri-carb 3100TR, Isotech, Chesterfield, UK).

#### 2.3.2.4 <sup>33</sup>P tissue analysis

Freeze dried plant material was homogenised by grinding the material to a fine powder using a milling device (A10 basic, IKA mills, Oxford). To quantify <sup>33</sup>P, the samples (30-50mg plant or 40-100mg soil) were digested in 1 ml concentrated sulphuric acid at 365°C for 15 minutes. Upon cooling, 100 µl hydrogen peroxide (Acros Organics, Geel, Belgium) was added to the samples and reheated to 365°C for 1 minute and repeated until the solution cleared. The cleared sample was diluted up to 10 ml with distilled water. <sup>33</sup>P activity was quantified via Packard Tri-carb 3100TR (Isotech, Chesterfield, UK) using 2 ml of digest solution with 10 ml Emulsify-safe (Perkin-Elmer). <sup>33</sup>P has a half-life of 25.34 days therefore, to accurately measure transfer between symbionts at the time of the experiment the results were corrected for radioactive decay and measured using the following equation (Equation 1) (Cameron et al., 2007).

$$M_{33P} = \left\{ \left[ \frac{(C_{DPM}/60)}{S_{Act}} \right] M_{wt} \right\} D_f$$

**Equation 1:** Where M<sub>33P</sub> = mass of <sup>33</sup>P (mg), CDPM = counts as disintegrations per minute, S<sub>Act</sub> = specific activity of the source (Bq mmol<sup>-1</sup>), D<sub>f</sub> = dilution factor (in this case 10) and M<sub>wt</sub> = molecular mass (of P) (Taken from Cameron et al. 2007).

#### 2.3.2.5 <sup>14</sup>C tissue analysis

To measure <sup>14</sup>C present within the plant and soil material, between 10 and 40 mg of the sample was placed in a Combusto-cone (Perkin Elmer) and the <sup>14</sup>C content measured sample oxidation (Model 307 Packard Sample Oxidiser; Isotech, Chesterfield, UK). CO<sub>2</sub> released through oxidation was trapped in 10 ml CarbonTrap (Meridan Biotechnologies) and mixed with 10 ml CarbonCount (Meridan Biotechnologies). Radioactivity present in the sample measured (Packard Tri-carb 3100TR, Isotech, Chesterfield, UK). The mass of <sup>14</sup>C was quantified using the following equation (Cameron et al., 2006) (Equation 2).

$$M_{14c} = \left( \frac{C_{DPM}/60}{SP_{Act}} \right) M_{wt}$$

**Equation 2:** ( $M_{14C}$ , the mass of  $^{14}C$  (mg);  $C_{DPM}$ , counts as disintegrations per minute;  $SP_{Act}$ , the specific activity of the source ( $Bq\ mmol^{-1}$ );  $M_{wt}$ , molecular mass (of C) (Cameron et al., 2006)).

Total C ( $^{12}C + ^{14}C$ ) transferred from the wheat plants to their AMF fungal partners was calculated by quantifying the mass of  $CO_2$  content in the labelling chamber and the proportion of the supplied  $^{14}CO_2$  which was fixed by the plants, see equations below (Equation 3, Equation 4) (Cameron et al., 2008). The difference in total C between the static and rotated core represents C transfer from the wheat plants to AMF within the cores. The C transfer in the cores was scaled up to AMF in the whole pot by multiplying the C per gram of soil with the biomass of the bulk soil in the pot.

$$T_{pf} = \left( \left( \frac{A}{A_{sp}} \right) m_a \right) + (P_r \times m_c)$$

**Equation 3:** ( $T_{pf}$ , total carbon transferred from plant to fungus in any given pool (g);  $A$ , radioactivity (Bq);  $A_{sp}$ , specific activity of the source ( $Bq\ Mol^{-1}$ ); and  $m_a$ , atomic mass of the element, in this case the isotope  $^{14}C$ ;  $P_r$ , proportion of the total  $^{14}C$  label supplied (as  $^{14}CO_2$ ) that is present in the tissue;  $m_c$ , mass of C in the  $CO_2$  present in the labelling chamber in  $m^3$  (from the ideal gas law, Eqn 4) (Taken from Cameron et al., 2008))

$$m_{cd} = M_{cd} \left( \frac{PV_{cd}}{RT} \right) \text{ thus } m_c = m_{cd} \times 0.27292$$

**Equation 4:** ( $m_{cd}$ , mass of  $CO_2$ ;  $M_{cd}$ , molecular mass of  $CO_2$ ;  $P$ , pressure;  $V_{cd}$ , volume of  $CO_2$  in the chamber ( $V_{cd} = 0.044\%$  of the total chamber volume based on an atmospheric  $[CO_2]$  of 440 ppm or 800 ppm);  $m$ , mass of unlabelled C in the labelling chamber;  $M$ , molar mass ( $M$  of C = 12.011 g);  $R$ , universal gas constant;  $T$ , absolute temperature;  $m_c$ , mass of C in the  $CO_2$  present in the labelling chamber in  $m^3$ ; 0.27292 represents the proportion of C in  $CO_2$  on a mass fraction basis (Taken from Cameron et al., 2008)).

### 2.3.2.6 <sup>15</sup>N tissue analysis

To determine <sup>15</sup>N abundance within the shoot tissue the freeze-dried and homogenised material was weighed (2-4 mg) into 6 x 4 mm<sup>2</sup> tin capsules (Sercon Ltd.). The samples were analysed using a continuous flow IRMS (PDZ 2020 IRMS, Sercon Ltd), with air used as the reference standard. Percentage N and atom percentage <sup>15</sup>N of the samples was measured. <sup>15</sup>N transferred from fungus to plant was calculated using the following equation (Equation 5) (Cameron et al., 2006).

$$M_{Ex} = \left( \frac{At_{lab} - At_{cont}}{100} \right) \left( M \left[ \frac{\%N}{100} \right] \right)$$

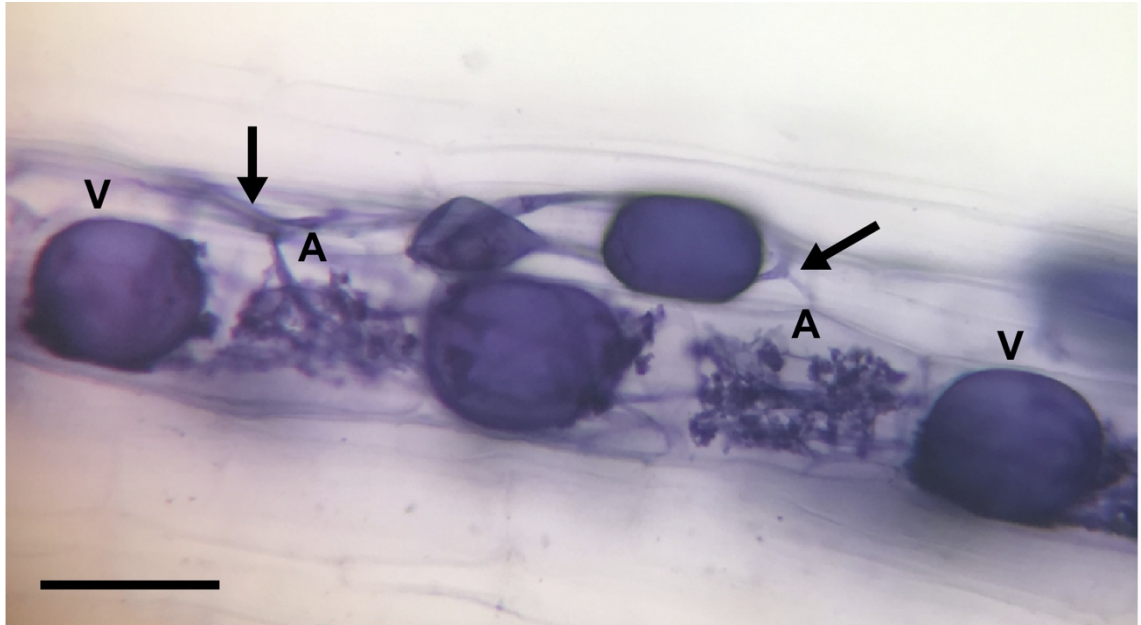
**Equation 5:** ( $M_{Ex}$ , mass (excess) of the isotope in g;  $At_{lab}$ , atom percentage of the isotope in labelled microcosm;  $At_{cont}$ , atom percentage of the isotope in paired control microcosm,  $M$ , biomass of sample (g) and  $\%N$ , percentage of nitrogen (Taken from Cameron et al., 2006)).

### 2.3.3 Mycorrhizal root colonisation

Representative subsamples of the roots collected from different areas in the root system upon plant harvest were placed in micromesh biopsy cassettes (Simport, Beloeil, Canada). To clear the roots, they were submerged in a container with preheated 10% KOH at 80 °C for 45 minutes. The cassettes containing cleared roots were rinsed with water and submerged in a second container with an ink and vinegar staining solution for 20 minutes at room temperature (Vierheilg et al., 1998). The staining solution consisted of 5% of brilliant black ink (Pelikan, Berlin, Germany) and 5% of acetic acid (Merck, Darmstadt, Germany), and 90% of distilled water. After staining the histology cassettes were rinsed with water, to remove any excess stain. Roots were stored in 1% acetic acid until they were mounted on microscope slides.

From the stained root subsample, 20 root fragments were picked at random and mounted on a glass microscope slide with a polyvinyl-lactoglycerol (PVLG) mountant and set in a 60 °C oven overnight. The PVLG mountant contained 16.6 g of polyvinyl alcohol dissolved in 10 ml Glycerol, 100 ml Lactic acid and 100 ml dH<sub>2</sub>O.

Mycorrhizal colonisation was assessed using the gridline intersect method with at least 100 lines intersects measured per root system (McGonigle et al., 1990). Each line intersect was scored for the presence of AMF hyphae, arbuscules and vesicles crossing the gridline on the microscope eyepiece (McGonigle et al., 1990). Out of the 100 intersections percentage of the root colonised by AMF, percentage arbuscules and percentage vesicles could be calculated.



**Figure 2.4: Stained root of wheat (*Triticum aestivum*) colonised by the arbuscular mycorrhizal fungus *Rhizophagus irregularis*.** The image demonstrates typical intracellular fungal structures, vesicles (V) and arbuscules (A) with interconnecting hyphae (arrows). Bar, 50  $\mu$ m.

#### **2.3.4 Hyphal extractions and measuring extraradical hyphal lengths**

A subsample of soil (10-20 g) was collected upon plant harvest and stored at 4 °C until extraction. Between 4-6 g of the subsample was weighed out and the exact mass recorded, this was then oven dried (80 °C) and the dry soil mass was recorded. From these values, the proportion of the fresh mass to dry mass was calculated. This proportion was then used to determine the dry mass of the extraction sample. The fresh mass of the extraction sample was recorded (1-3 g). The sample was placed in a beaker of water (1000 ml) and mixed thoroughly on a magnetic stirring plate. The magnetic stirrer was turned off but as the liquid was still spinning 200 ml was poured off into a smaller beaker and returned to the stirrer plate. After 30 seconds the stirrer was again turned off, and 10ml of the spinning liquid was extracted from the centre of the beaker where the hyphae

should be accumulating, using a syringe. This liquid was then dispensed into the Millipore filtration apparatus attached to a vacuum pump. The fluid was drawn through a filter paper disc which catches the hyphae; trypan blue stain was used to dye the hyphae and washed off with distilled water. Two filter paper discs per plant replicate (n=24) were mounted on slides with polyvinyl-lactoglycerol and set in a 60 °C oven overnight.

Hyphal lengths were quantified by using a graticule microscope eyepiece with a 10x10 grid. The hyphae collected on the membrane filter paper were viewed under the grid to measure how many times hyphae intersects with a line on the grid. A hand tally counter was used to record root grid-line intersections. Two hundred fields of view were examined per microscope slide at x 400 magnification.

### **2.3.5 Measuring total phosphorus**

To quantify total P within the plant material the samples of homogenised root, shoot and grain material (30-50 mg) were digested in 1 ml concentrated sulphuric acid at 365°C, as described above.

A standard curve of known P concentrations (ppm) was used to calculate the P concentrations (ppm) in the unknown samples. A 10 ppm stock solution for P standards was made by dissolving 44.55 mg of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (sodium dihydrogen orthophosphate, M.W. 137.99) in 1 L of  $\text{dH}_2\text{O}$ . The stock solution was used to make P standards which ranged from 0-8 ppm. Optical density for known [P] was measured at 882 nm on a spectrophotometer. The cuvettes contained 10 ppm P stock solution (0-0.4 ml), 0.2 ml of the acid digest blank, 0.5 ml ammonium molybdate reagent, and 0.2 ml of 0.1 M L-ascorbic acid, made up to 3.8 ml by  $\text{dH}_2\text{O}$  within a cuvette and left to develop for 45 minutes. A standard curve was produced using the known P values and associated the optical density values, if an  $R^2$  value of between 0.98-1 was achieved the curve was used for accurate colourimetric determination of [P] in the unknown samples (Murphy & Riley, 1962; Leake, 1988).

Quantification of P in unknown samples required 0.2 ml of the acid digested plant sample, 0.5 ml ammonium molybdate reagent, and 0.2 ml of 0.1 M L-ascorbic acid made up to 3.8 ml by  $\text{dH}_2\text{O}$  within a cuvette. The solution was left to develop for 45 minutes then optical density was measured at 882 nm on a



spectrophotometer. The optical density values for the unknown samples were, converted to P (ppm) using the equation of the trend line on the standard curve.

$$y = mx + b$$

**Equation 6:** y, phosphorus concentration in ppm; m, is the slope of the line; x, the optical density of the unknown sample; b, is where the line intercepts the y-axis.

### 2.3.6 Statistical analysis

The impact of AMF inoculant treatment on measured parameters, and whether effects differed between three wheat cultivars was assessed by two-way ANOVA followed by Tukey HSD tests using Minitab (Version 17). Before statistical analysis, data were checked for confirmation to assumptions of normality and equal variance using normal probability plots and residuals vs fits plots.

In the cases of shoot biomass, grain biomass, arbuscules, vesicles, extra-radical hyphal length, total shoot P, root [P], total root P, grain [P], fungal C in the core, fungal C in the pot and % C allocated to fungi in the core the data was transformed using the optimal lambda function in Minitab (Version 17), to fulfil data assumptions.

The homoscedasticity of the concentration and total <sup>33</sup>P data in the shoots and the roots could not be fixed by transforming the data. Therefore, individual Student's t-tests were performed between AMF treatments within each wheat cultivar on Minitab (Version 17). A one-sample t-test was conducted for cv. Cadenza root <sup>33</sup>P (ng), with control treatment (not inoculated with *R. irregularis*) tested against a hypothesised mean of zero, as the treatment inoculated with *R. irregularis* produced all zero values, which was not compatible with a two-sample t-test in Minitab (Version 17).

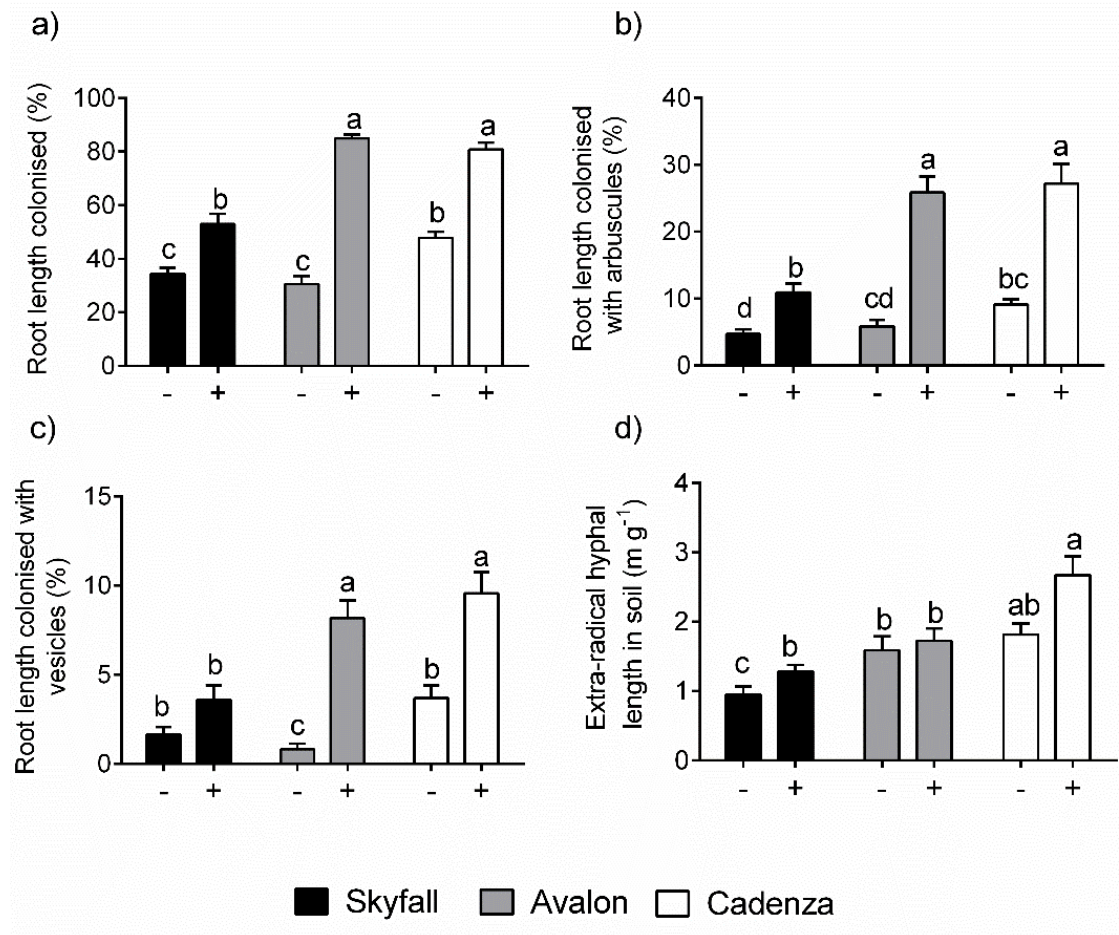
## 2.4 Results

### 2.4.1 Root colonisation and extra-radical hyphal lengths.

AMF colonisation was well established (between 11% and 93%) within wheat root systems in all cultivars, across all treatments. Inoculation of plants with *R. irregularis* resulted in a large increase in mycorrhizal colonisation within the roots above the colonisation present when they were grown in agricultural soil only. In plants which were not inoculated with additional *R. irregularis*, colonisation within the cultivars varied between a mean of 31%, 34% and 48% in cv. Avalon, Skyfall and Cadenza respectively. When the plants were inoculated with *R. irregularis*, the extent of the increase in colonisation was different between the wheat cultivars (Interaction:  $F=22.17$ ,  $d.f.=2,66$ ,  $P<0.001$ ; two-way ANOVA, Table 2.2). cv. Skyfall had the smallest rise in root colonisation of the wheat varieties (54.4%), followed by cv. Cadenza (68.9% increase) with cv. Avalon having the most substantial increase in colonisation (177.9%).

**Table 2.2:** Summary of two-way ANOVA (GLM) results testing the effects of inoculation with *R. irregularis* on three wheat cultivars. Bold indicates significant differences, where interactions were significant main effects were not reported.

<b>Variable</b>	<b>AMF treatment</b>		<b>Wheat cultivar</b>		<b>AMF x Cultivar</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	1, 66	<i>d.f</i>	2,66	<i>d.f</i>	2,66
Root colonisation (%)	257.2	<0.001	30.6	<0.001	22.2	<b>&lt;0.001</b>
Arbuscules (%)	136.5	<0.001	23.5	<0.001	7.0	<b>0.002</b>
Vesicles (%)	72.0	<0.001	14.0	<0.001	7.3	<b>0.001</b>
ER hyphae length (m g <sup>-1</sup> )	10.2	<b>0.002</b>	22.2	<b>&lt;0.001</b>	1.05	0.355



**Figure 2.5: Percentage of the root length colonised by arbuscular mycorrhizal fungi with or without inoculation with *R. irregularis*.** (a) total colonisation, (b) arbuscules, (c) vesicles and (d) extraradical hyphal lengths in the soil. Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and Cadenza (white bars). Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Error bars represent the standard error of the mean. Different letters refer to significant differences (n=12,  $P < 0.05$ , two-way ANOVA, TukeyHSD post-test) between treatments.

The presence of fungal structures, arbuscules and vesicles, also increased within the root system of wheat plants inoculated with *R. irregularis*. When the plants were grown in agricultural soil only, cv. Cadenza had the highest proportion of arbuscules out of the three wheat cultivars (Figure 2.5b). The mean percentage of the root length colonised with arbuscules in each cultivar was 5%, 6% and 9% in cv. Skyfall, Avalon and Cadenza respectively. When inoculated, both cv. Cadenza (27%) and cv. Avalon (26%) had the highest abundance of arbuscules, with cv. Skyfall (11%) having significantly fewer; so the magnitude of the

response to *R. irregularis* inoculum was cultivar dependent (Interaction: ANOVA:  $F=7.0$ , d.f.=2,66,  $P<0.01$ ; two-way ANOVA, Table 2.2).

Vesicles were a less prominent structure in the root system, in plants grown in agricultural soil only they were only present in 2% (cv. Skyfall), 1% (cv. Avalon) and 4% (cv. Cadenza) of the root length. The presence of vesicle structures increased significantly to 8% of the root length in cv. Avalon and 10% in cv. Cadenza when *R. irregularis* inoculum was added. In cv. Skyfall there was a small but non-significant increase (4%), revealing the response to *R. irregularis* inoculation to be a cultivar-dependent interaction (Interaction: ANOVA:  $F=7.3$ , d.f.=2,66,  $P<0.01$ ; two-way ANOVA, Table 2.6, Figure 2.5c). However, increases in percentage root length colonisation (and arbuscules/vesicles) in both cv. Cadenza and Avalon are likely to be overestimated due to concurrent decreases in root biomass (Figure 2.10b).

The length of the extra-radical fungal hyphae extracted from the soil did not increase significantly within individual cultivars when the plants were inoculated with AMF (Figure 2.5d) (Tukey:  $P>0.05$ ). Overall, inoculation did increase the presence of hyphae in the soil significantly ( $F=10.24$ , d.f.=1,66,  $P<0.01$ ; two-way ANOVA, Table 2.6). Hyphal lengths in the soil also differed depending on the wheat cultivar ( $F=22.18$ , d.f.=22,66,  $P<0.001$  two-way ANOVA, Table 2.2), cv. Cadenza had the most hyphae per gram of soil followed by cv. Avalon and then cv. Skyfall (Figure 2.5d).

#### **2.4.2 The effect of AMF inoculation on plant nutrient uptake.**

Adding *R. irregularis* inoculum increased shoot tissue [P] in all wheat cultivars compared to the control plants in agricultural soil only. The magnitude of the increases differed between cultivars (Interaction:  $F=5.54$ , d.f.=2,66,  $P<0.01$ ; two-way ANOVA, Table 2.6). [P] in the shoots increased by 14.3%, 32.4% and 18.2%, in cv. Skyfall, Avalon and Cadenza respectively (Figure 2.6a). Total amounts of P in the shoot material also increased in all cultivars, when plants were inoculated with *R. irregularis* ( $F=41.65$ , d.f.=1,66,  $P<0.001$ ; two-way ANOVA, Table 2.6, data not shown).

Within the root system [P] increased significantly when the plants were inoculated with *R. irregularis* (Figure 2.6b) ( $F=48.0$ , d.f.=1,64,  $P<0.001$ ; two-way ANOVA, Table 2.6). In cv. Avalon and Cadenza, a decrease in root biomass correlated to

an increase in root [P], but no increase in overall P accumulation in the tissue. In contrast, there was a significant increase in total P within the root system in cv. Skyfall (Interaction:  $F=5.4$ ,  $d.f.=2,66$ ,  $P<0.01$ ; two-way ANOVA, Table 2.6, data not shown).

**Table 2.3:** Summary of two-way ANOVA (GLM) results testing the effects of inoculation with *R. irregularis* on phosphorus assimilation in three wheat cultivars. Bold indicates significant differences, where interactions were significant main effects were not reported.

<b>Variable</b>	<b>AMF treatment</b>		<b>Wheat cultivar</b>		<b>AMF Cultivar x</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	1, 66	<i>d.f</i>	2, 66	<i>d.f</i>	2, 66
Shoot [P] (mg g <sup>-1</sup> )	77.7	<0.001	16.3	<0.001	5.5	<b>0.006</b>
Shoot P (mg)	41.7	<0.001	251.2	<0.001	4.0	<b>0.023</b>
Root [P] (mg g <sup>-1</sup> )	48.0	<b>&lt;0.001</b>	7.8	<b>0.001</b>	1.9	0.157
Root P (mg)	1.0	0.331	73.9	<0.001	5.4	<b>0.007</b>

#### 2.4.2.1 <sup>33</sup>P transfer from fungus-to-plant

The functionality of the fungal hyphal networks in the soil cores was measured by determining their uptake and delivery of <sup>33</sup>P from the cores to the wheat. There was no increase in the concentration of <sup>33</sup>P in the plant shoot material when plants were inoculated with *R. irregularis* in any of the wheat cultivars. (Figure 2.6c). cv. Avalon received significantly more <sup>33</sup>P when grown in agricultural soil only with the native AMF community, than when additionally inoculated with commercial *R. irregularis* inoculum ( $t=3.34$ ,  $d.f. =8$ ,  $P=0.01$ ; Student's t-test, Table 2.4). Whereas, in cv. Skyfall and cv. Cadenza there was no significant difference in <sup>33</sup>P acquired from an AMF partner when they were inoculated with *R. irregularis* compared to when plants were in the presence of the native AMF community only (cv. Skyfall –  $t= 0.45$ ,  $d.f.=9$ ,  $P=0.66$ ; Student's t-test, Table 2.4), (cv. Cadenza- $t=0.25$ ,  $d.f.=7$ ,  $P=0.81$ ; Student's t-test, Table 2.4).

There was no significant increase in the concentration or total amount of mycorrhizal acquired <sup>33</sup>P in the root system when the plants were inoculated with *R. irregularis* (Figure 2.6d). Similarly to in the shoot material, there was a significant decrease in <sup>33</sup>P in the root tissue of cv. Avalon (both total and concentration) (Table 2.4). There were differences in the mycorrhizal acquired

$^{33}\text{P}$  depending on the wheat cultivar, cv. Skyfall generally received more  $^{33}\text{P}$  from an AMF partner than the other wheat cultivars (Figure 2.6c,d).

**Table 2.4:** Summary of t-test results testing the effects of inoculation with *R. irregularis* on mycorrhizal-acquired  $^{33}\text{P}$  in three wheat cultivars. Bold indicates significant differences.

Variable	Skyfall			Avalon			Cadenza		
	<i>T</i>	<i>P</i>	<i>DF</i>	<i>T</i>	<i>P</i>	<i>DF</i>	<i>T</i>	<i>P</i>	<i>DF</i>
Shoot [ $^{33}\text{P}$ ] (ng g <sup>-1</sup> )	0.45	0.66	9	3.34	<b>0.01</b>	8	0.25	0.81	7
Shoot $^{33}\text{P}$ (ng)	1.09	0.33	5	2.66	<b>0.03</b>	9	1.54	0.16	8
Root [ $^{33}\text{P}$ ] (ng g <sup>-1</sup> )	1.88	0.09	9	3.52	<b>0.01</b>	8	2.02	0.10	5
Root $^{33}\text{P}$ (ng)	0.72	0.50	8	3.66	<b>0.02</b>	5	1.58	0.18	NA*

\* A one-sample t-test was conducted for cv. Cadenza root  $^{33}\text{P}$  (ng), with control treatment (not inoculated with *R. irregularis*) tested against a hypothesised mean of zero, as the treatment inoculated with *R. irregularis* produced all zero values.

#### 2.4.2.2 Nitrogen concentrations and $^{15}\text{N}$ transfer from fungus-to-plant

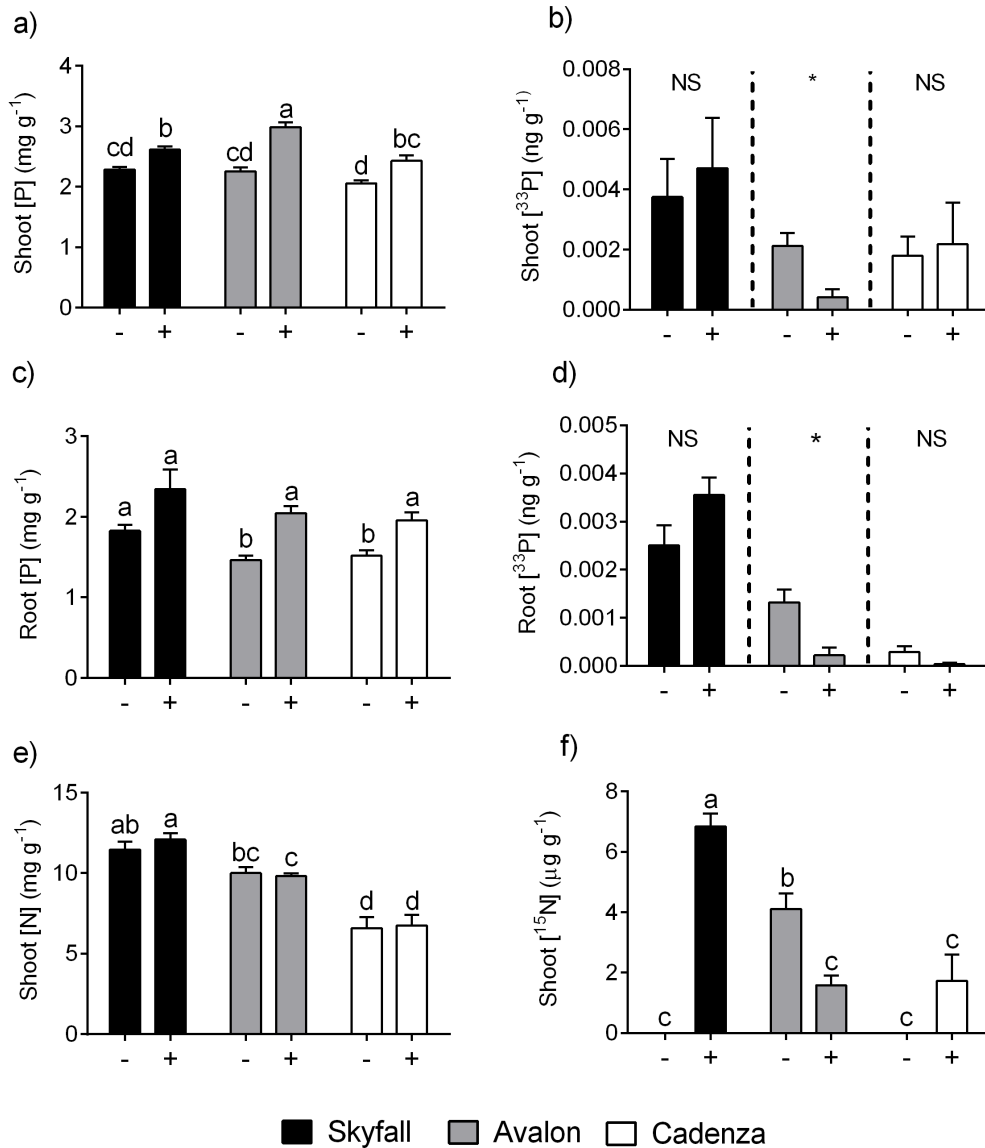
Inoculation with AMF did not increase N concentrations ([N]) ( $F=0.13$ , d.f.=1,54,  $P=0.718$ ; two-way ANOVA, Table 2.6) or total N within the shoot tissue ( $F=0.91$ , d.f.=1,54,  $P=0.344$ ; two-way ANOVA, Table 2.5, Figure 2.6e). Cultivars differed in their tissue [N], with cv. Skyfall having the highest concentrations of N at an average of 11.8 mg g<sup>-1</sup> which is 19% more than cv. Avalon (9.9 mg g<sup>-1</sup>) and 56% more than cv. Cadenza (7.5 mg g<sup>-1</sup>) ( $F=64.5$ , d.f.=2,54,  $P<0.001$ ; two-way ANOVA, Table 2.5). The increase in P but not N in the shoot material, when plants were inoculated with *R. irregularis* caused a decrease in the N:P ratios of the plant material ( $F=31.6$ , d.f.=1,54,  $P<0.001$ ; two-way ANOVA, Table 2.5, Figure 2.7).

The functionality of the fungal hyphal networks in the soil cores, was also determined by measuring their ability to uptake and deliver  $^{15}\text{N}$  to wheat. The effect of inoculation with *R. irregularis* on  $^{15}\text{N}$  delivery varied massively depending on wheat cultivar (Interaction:  $F=51.0$ , d.f.= 2,30,  $P<0.001$ ; two-way ANOVA, Table 2.5, Figure 5f). In soil-only (i.e. without additions of AMF inoculum) there

was no mycorrhizal acquired  $^{15}\text{N}$  in cv. Skyfall, however, when the soil was inoculated with *R. irregularis*, mycorrhizal acquired  $^{15}\text{N}$  increased to the highest level of all treatments at  $6.8 \mu\text{g g}^{-1}$ . On the other hand, there was a significant decrease in mycorrhizal acquired  $^{15}\text{N}$  when cv. Avalon was inoculated with *R. irregularis*, compared to uninoculated soil-only plants, from  $4.1 \mu\text{g g}^{-1}$  to  $1.6 \mu\text{g g}^{-1}$ . cv. Cadenza had the lowest concentration of mycorrhizal acquired  $^{15}\text{N}$  in the shoot material and was not significantly affected by the addition of *R. irregularis* inoculum.

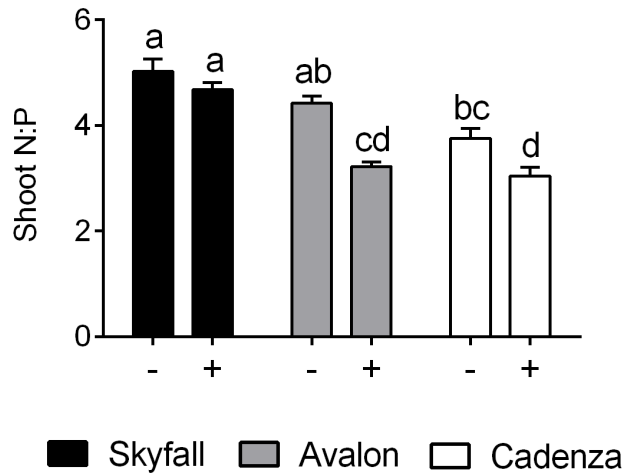
**Table 2.5:** Summary of two-way ANOVA (GLM) results testing the effects of inoculation with *R. irregularis* on nitrogen assimilation in three wheat cultivars. Bold indicates significant differences, where interactions were significant main effects were not reported.

<b>Variable</b>	<b>AMF treatment</b>		<b>Wheat cultivar</b>		<b>AMF x Cultivar</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f.</i>	1, 30	<i>d.f.</i>	2, 30	<i>d.f.</i>	2, 30
Shoot [ $^{15}\text{N}$ ] ( $\mu\text{g g}^{-1}$ )	28.3	<0.001	16.7	<0.001	51.0	<b>&lt;0.001</b>
Shoot $^{15}\text{N}$ ( $\mu\text{g}$ )	19.0	<0.001	27.5	<0.001	48.6	<b>&lt;0.001</b>
<i>Degrees of freedom</i>	<i>d.f.</i>	1, 54	<i>d.f.</i>	2, 54	<i>d.f.</i>	2, 54
Shoot [N] ( $\text{mg g}^{-1}$ )	0.1	0.718	64.5	<b>&lt;0.001</b>	1.0	0.377
Shoot N (mg)	0.91	0.344	25.5	<b>&lt;0.001</b>	1.6	0.205
N:P	31.6	<0.001	41.4	<0.001	3.3	<b>0.043</b>



**Figure 2.6: Phosphorus and nitrogen assimilation by the host plant and arbuscular mycorrhizal fungi with or without inoculation with *R. irregularis*.** (a) Plant shoot tissue [P], n=12. (b) Plant shoot tissue [<sup>33</sup>P] assimilated via fungal symbionts, n=6. (c) Plant root tissue [P], n=12. (d) Plant root tissue [<sup>33</sup>P] assimilated via fungal symbionts, n=6. (e) Plant shoot tissue [N], n=10. (f) Fungal-assimilated [<sup>15</sup>N] in plant shoot tissues, n=6. Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and Cadenza (white bars). Error bars represent the standard error of the mean. Different letters refer to significant differences between treatments. \* refers to significant differences between treatments. ns refers to no significant differences. (a, b, e, f),  $P < 0.05$ , two-way ANOVA, TukeyHSD posthoc test. (c, d) Student's t-tests,  $P < 0.05$ .





**Figure 2.7: N:P of shoot tissues grown in an agricultural soil with or without inoculation with *R. irregularis*.** Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and cadenza (white bars). Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Error bars represent the standard error of the mean. Different letters refer to significant differences between treatments.  $n=10$ ,  $P<0.05$ , two-way ANOVA, TukeyHSD post-test.

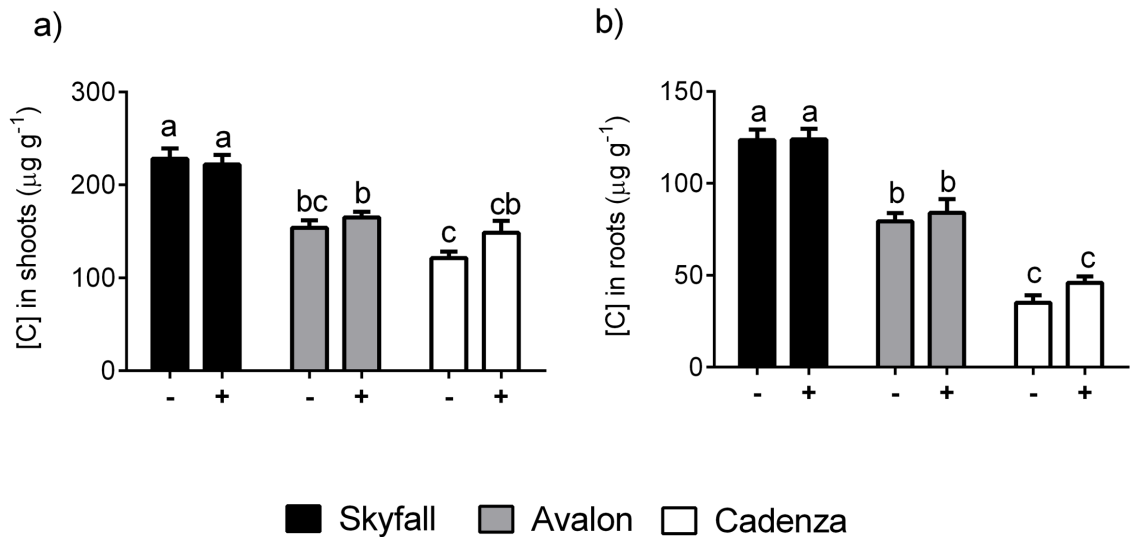
### 2.4.3 The effect of AMF inoculation on plant carbon allocation to the fungal partner.

Total C acquired by the plants over a 16-hour photoperiod was determined using  $^{14}\text{CO}_2$ . There were apparent cultivar differences in the total amount ( $F=5.04$ , d.f.=2,65,  $P<0.01$ ; two-way ANOVA, Table 2.6) and concentration ( $F=49.43$ , d.f.=2,65,  $P<0.001$ ; two-way ANOVA, Table 2.6) of C the plants accumulated in their shoot tissue over this time. cv. Skyfall had the highest concentration of C in its shoot tissue (Figure 2.8a), whereas, cv. Cadenza accumulated the highest total amount, likely influenced by the cultivar differences in shoot biomass presented below (Figure 2.10a). There were also apparent cultivar differences in the total amounts of C acquired by the root material, with cadenza having a four-fold lower C accumulation than the other cultivars. cv. Skyfall had the highest concentration of C in the root system ( $123 \mu\text{g g}^{-1}$ ) followed by cv. Avalon which had 34% less, then cv. Cadenza which had 67% less (Figure 2.8b). However,

inoculation of the plants with *R. irregularis* did not affect the total amount or concentration of C assimilated within the plant tissue (Table 2.6, Figure 2.8a,b).

**Table 2.6:** Summary of two-way ANOVA (GLM) results testing the effects of inoculation with *R. irregularis* on carbon allocation in three wheat cultivars. Bold indicates significant differences.

<b>Variable</b>	<b>AMF treatment</b>		<b>Wheat cultivar</b>		<b>AMF x Cultivar</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	1, 66	<i>d.f</i>	2,66	<i>d.f</i>	2,66
C allocation to fungi in cores (ng)	1.0	0.316	4.9	<b>0.010</b>	0.4	0.675
C allocation to fungi in pot (µg)	0.4	0.558	5.0	<b>0.009</b>	0.4	0.692
<i>Degrees of freedom</i>	<i>d.f</i>	1, 65	<i>d.f</i>	2,65	<i>d.f</i>	2, 65
% C allocation to cores	0.0	0.985	1.5	0.222	0.2	0.843
[C] assimilation in the roots (µg g <sup>-1</sup> )	1.44	0.235	118.3	<b>&lt;0.001</b>	0.47	0.625
[C] assimilation in the shoots (µg g <sup>-1</sup> )	2.0	0.163	49.4	<b>&lt;0.001</b>	1.6	0.210

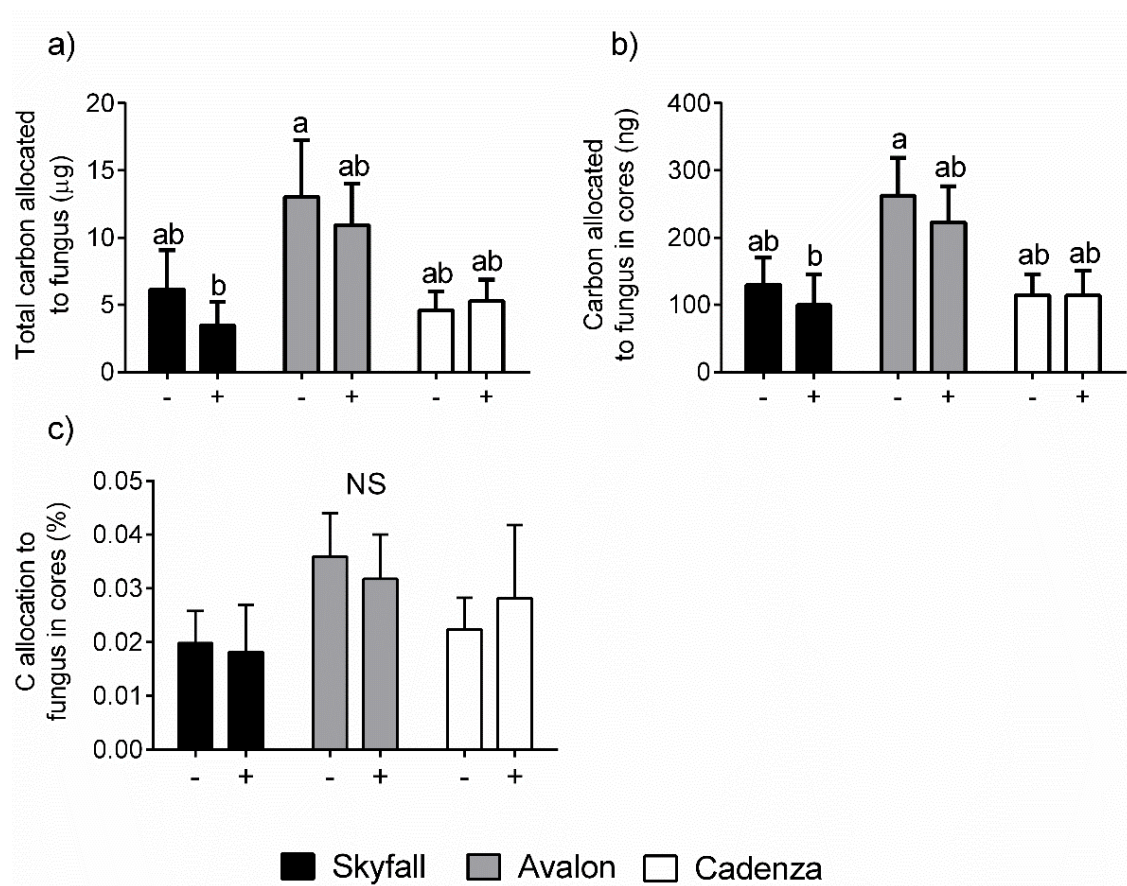


**Figure 2.8: Carbon assimilated in the plant shoot and root material over one photoperiod with or without inoculation with *R. irregularis*.** (a) The total [C] in the shoot material. (b) The total [C] in the root material. Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and Cadenza (white bars). Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Error bars represent the standard error of the mean. Different letters refer to significant differences between treatments. n=12,  $P < 0.05$ , two-way ANOVA, TukeyHSD post-test.

When C allocation to the fungal hyphae in the soil cores was examined, there was no significant difference between plants inoculated with *R. irregularis* and control plants in agricultural soil only in any of the cultivars ( $F=1.0$ , d.f.= 1,66,  $P=0.35$ ; two-way ANOVA, Table 2.6, Figure 2.9b). However, cultivar differences were observed in the amount of C allocated to the fungal hyphae within the soil cores, with cv. Avalon allocating more than double the amount of C to its fungal partner than cv. Skyfall or cv. Cadenza ( $F=4.9$ , d.f.=2,66,  $P=0.01$ ; two-way ANOVA, Table 2.6).

The percentage of C allocated to the fungi within the soil cores amounted to less than 0.05% of total assimilated C in all wheat cultivars (Figure 2.9c). Calculations were used to scale up the allocation of C to AMF hyphae within the soil core to the all soil hyphae within the experimental pot (see section 2.3.2.5) (Figure 2.9a). The percentage of total fixed C the wheat cultivars allocated to the AMF within a

photoperiod was still negligible and dependant on cultivar treatment combination, with the highest being in cv. Avalon at 2%.

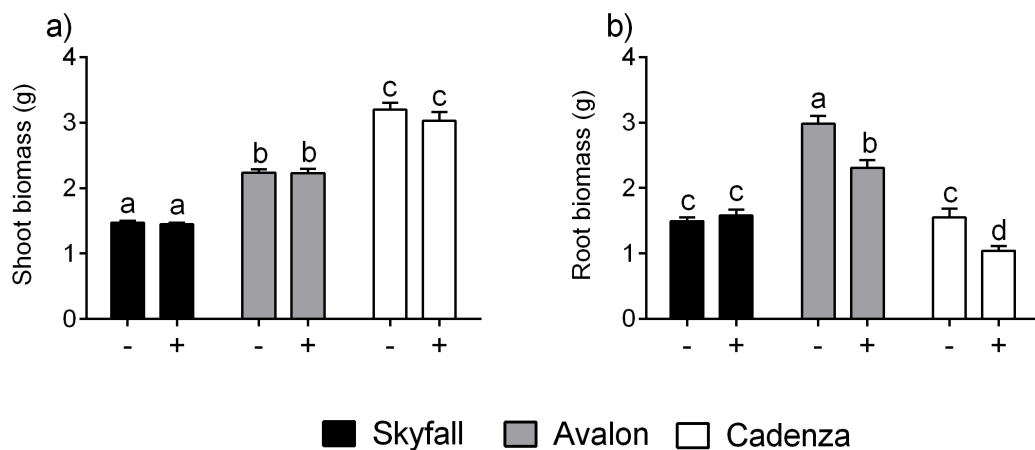


**Figure 2.9: Carbon transfer from wheat plants to their fungal partners over one photoperiod with or without inoculation with *R. irregularis*.** (a) Total measured plant-fixed carbon transferred to fungi in soil. (b) Total measured plant-fixed carbon transferred to fungi in cores. (c) Percentage allocation of plant-derived carbon to fungi within cores. Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and Cadenza (white bars). Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Error bars represent the standard error of the mean. Different letters refer to significant differences between treatments (n=12,  $P < 0.05$ , two-way ANOVA, TukeyHSD post-test, data transformation by optimal lambda).

#### 2.4.4 The effect of AMF inoculation on plant biomass and yield

Shoot biomass differed significantly between wheat cultivars after eight weeks of growth. The use of *R. irregularis* inoculant had no impact on the wheat shoot biomass compared to the control plants in agricultural soil only ( $F = 1.15$ , d.f. = 1,66,  $P > 0.05$ ; two-way ANOVA, Table 2.7, Figure 2.10a). Root system sizes also

differed significantly between cultivars with cv. Avalon having the most extensive root system and cv. Cadenza having the smallest. Variation in the size of the shoot and root systems between the varieties led to root: shoot ratio differences, with cv. Cadenza having a much lower allocation to its root system with an average root to shoot ratio of 0.42 compared to cv. Avalon and cv. Skyfall which had much higher values, 1.18 and 1.05 respectively. In two of the wheat cultivars (Avalon, Cadenza) inoculation with AMF caused a decrease in the biomass of the root system compared to the control plants, but there was no decrease in investment in the root system in cv. Skyfall (Interaction:  $F=7.38$ ,  $d.f.=2,66$ ,  $P=0.001$ ; two-way ANOVA, Table 2.7).



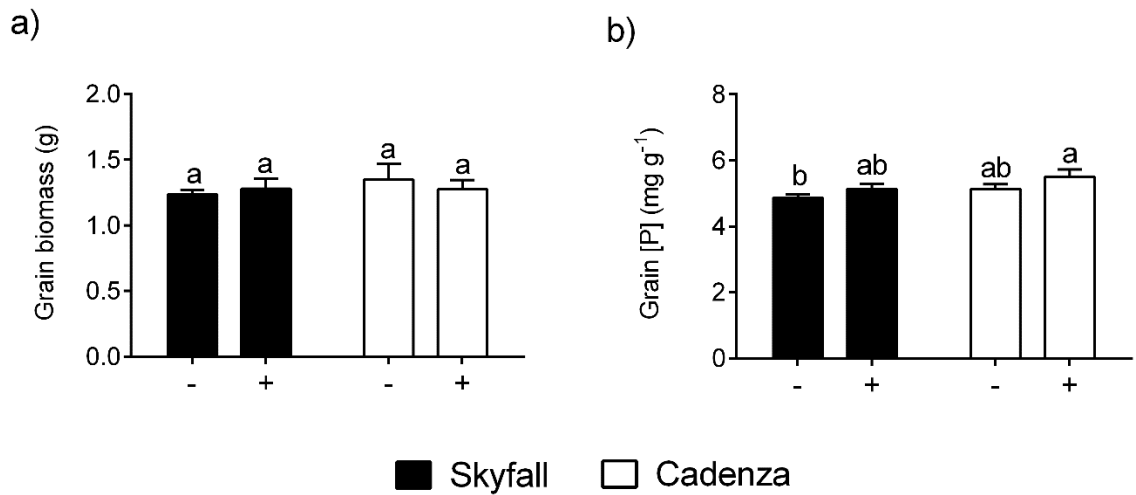
**Figure 2.10: Wheat plant biomass with or without inoculation with *R. irregularis*** (a) shoot biomass (b) root biomass. Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and cadenza (white bars). Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Error bars represent the standard error of the mean. Different letters refer to significant differences between treatments (n=12,  $P<0.05$ , two-way ANOVA, TukeyHSD post-test, shoot biomass data transformed by optimal lambda).

Wheat grain yield was measured in cv. Skyfall and cv. Cadenza and was similar between the two wheat cultivars and unaffected by inoculation with *R. irregularis* ( $F=0.89$ ,  $d.f.=1,28$ ,  $P>0.05$ ; two-way ANOVA, Table 2.7, Figure 2.11a). The concentration of P within the wheat grain was higher overall in the plants treated

with *R. irregularis* commercial inoculum ( $F=5.05$ ,  $d.f.=1,28$ ,  $P<0.05$ ; two-way ANOVA, Table 2.7, Figure 2.11b), although there were no significant differences within the individual cultivars (Tukey,  $P>0.05$ ).

**Table 2.7:** Summary of two-way ANOVA (GLM) results testing the effects of inoculation with *R. irregularis* on biomass in three wheat cultivars. Bold indicates significant differences, where interactions were significant main effects were not reported.

<b>Variable</b>	<b>AMF treatment</b>		<b>Wheat cultivar</b>		<b>AMF x Cultivar</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f.</i>	1, 66	<i>d.f.</i>	2,66	<i>d.f.</i>	2,66
Shoot biomass (g)	1.2	0.287	392.9	<b>&lt;0.001</b>	0.24	0.784
Root biomass (g)	19.0	<0.001	97.8	<0.001	7.4	<b>0.001</b>
<i>Degrees of freedom</i>	<i>d.f.</i>	1,28	<i>d.f.</i>	1,28	<i>d.f.</i>	1,28
Grain biomass (g)	0.02	0.890	0.31	0.585	0.34	0.562
Grain [P] (mg g <sup>-1</sup> )	5.05	<b>0.033</b>	4.79	<b>0.037</b>	0.27	0.605



**Figure 2.11: Wheat grain yield biomass and [P] with or without inoculation with *R. irregularis*** (a) Grain biomass (b) Grain [P]. Experiments were conducted in two wheat cultivars, Skyfall (black bars) and Cadenza (white bars). Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Error bars represent the standard error of the mean. Different letters refer to significant differences between treatments (n=8,  $P<0.05$ , two-way ANOVA, TukeyHSD post-test, data transformed by optimal lambda).

## 2.5 Discussion

### 2.5.1 Root colonisation and extra-radical hyphal lengths.

The significant increase in colonisation of wheat roots by AMF in pots where *R. irregularis* inoculum was added was likely caused by the inoculum, suggesting the inoculum itself was likely viable. Such increases in root colonisation observed in all the wheat cultivars tested could either be a direct result *R. irregularis* colonisation or caused by a facilitation effect of the inoculum, increasing colonisation by other species within the native AMF community. It is important not to presume that the increase in fungal colonisation in the root system observed here following inoculation with *R. irregularis* inoculum is exclusively *R. irregularis*, and this is a key area for future research to clarify (see Chapter 3).

The substantial rise in AMF colonisation in the plant roots when inoculated with *R. irregularis* In this study could be explained by low native AMF spore densities within the agricultural soil which may have been limiting the mycorrhizal colonisation of the non-inoculated plants (Lekberg & Koide, 2005). These results add to the growing evidence that AMF inocula may be an effective way of increasing AMF colonisation of crop plants in agricultural soil. Successful inoculation of wheat with *R. irregularis* has been shown previously where spore densities in the soil were initially very low (Mohammad et al., 2004). In addition, a meta-analysis of experiments conducted in non-sterile soil found that AMF inoculation was the most successful way of increasing mycorrhizal colonisation, over changes in management practices such as shorter fallow periods and reduced disturbance (Lekberg & Koide, 2005).

The increase in colonisation of roots in the present experiment varied widely between the wheat cultivars tested, ranging from 54 -178%. This suggests there may be differences in the ability of the particular strain of *R. irregularis* within the inoculum to colonise the different wheat cultivars. Previous studies have shown a tremendous difference in wheat cultivar colonisation with AMF (Zhu et al., 2001; Lehnert et al., 2017) which may be due to cultivar-AMF specificity.

Extra-radical hyphal networks are responsible for foraging in the soil for nutrients to pass to the plant partner and are in turn supported by the host plants C resources and therefore hyphal network size could be indicative of plant C



investment in a fungal partner. Extra-radical hyphal network length did not increase to the large extent seen in percentage root colonisation, although there was an overall significant effect of added inoculum on hyphal lengths. The substantial increase in root colonisation may not illustrate an improvement in the function of the symbiosis in terms of nutrient uptake by the plant partner (Smith et al., 2004; Li et al., 2005), whereas extra-radical hyphae lengths have more frequently been correlated with nutrient uptake (Jakobsen et al., 2001; Sawers et al., 2017; Svenningsen et al., 2018), but accurate measurements can be difficult and may have been restricted by pot volume.

Many studies use root colonisation as an indicator of function, assuming higher colonisation will indicate greater plant growth or nutrient uptake responses. However, within this experiment root colonisation is not a good indicator of function, and the presence of AMF structures does not necessarily indicate that those structures are translocating C, N or P (Fitter, 1991). For example, although root colonisation was higher in plants inoculated with *R. irregularis* compared to those grown in the presence of the native AMF community only, this did not translate into an increase in AMF-acquired P or N. Additionally, cultivar differences in the extent of root colonisation and AMF hyphae in the soil did not relate to the amount of AMF-acquired nutrients gained by the plants. Therefore, it is essential to measure the function of the symbiosis as the presence of AMF within the root system does not provide evidence of a functioning symbiosis. Beyond AMF inoculum being viable, we need it to increase nutrient uptake to have any potential to reduce chemical fertiliser additions.

## **2.5.2 The effect of AMF inoculation on plant nutrient uptake.**

### **2.5.2.1 Phosphorus**

As well as increasing root colonisation, adding *R. irregularis* inoculum increased tissue [P] in both the shoot and root material of all wheat cultivars tested. This substantial increase in plant P uptake of up to 32% when inoculated with *R. irregularis*, could be a vital tool for reducing P inputs in agriculture. Increases in tissue [P] have previously been shown in field studies of wheat inoculated with AMF (Mohammad et al., 2004), showing promise that the results in this experiment could be achieved in agricultural settings. The study involved four

levels of P fertilisation from 0-20 kg ha<sup>-1</sup> and showed plants inoculated with AMF receiving low fertiliser applications could equal or exceed the growth of non-inoculated plants which received higher fertiliser applications.

However, it is essential to directly measure mycorrhizal-acquired P as an increase in plant tissue P in the presence of an AMF inoculum cannot be accurately attributed to AMF function. The current study found that despite the increases in plant tissue P seen when plants were inoculated with *R. irregularis*, there was no corresponding increase in mycorrhizal acquired <sup>33</sup>P.

Discrepancies between mycorrhizal acquired P and increases in total plant P when plants are inoculated with AMF have been noted in other studies conducted in wheat. When comparing mycorrhizal wheat and non-mycorrhizal wheat, *G. intraradices* (now *R. irregularis*) supplied between 50-80% of the wheat's P while producing no increase in overall tissue [P] (Li et al., 2006). AMF partners supplying large amounts of plant P but producing no increase in overall tissue [P] suggest a complex interaction between the direct root uptake pathway and the AMF uptake pathway (Figure 1.1), highlighting that in Li et al., direct root uptake must have been reduced. In the current study, P uptake by the direct root pathway appears to be increased by the addition of commercial inoculum.

Most previous research into the effect of AMF on plant P assimilation indicates a downregulation in the direct pathway when plants associate with AMF (Pearson & Jakobsen, 1993a; Smith et al., 2003; Li et al., 2006; Facelli et al., 2014), some suggest that the effect of AMF on the direct uptake pathway is AMF species-specific (Pearson & Jakobsen, 1993a; Smith et al., 2000, 2003). AMF species *Scutellospora calospora* contributed only 7% of total P to a cucumber host which did not explain the 109% increase in P within the plant material when plants were inoculated with *S. calospora* compared to a non-mycorrhizal control (Pearson & Jakobsen, 1993). The more than doubling in tissue P in roots colonised by *S. calospora* despite only a small contribution of AMF to P uptake suggests that direct plant P uptake systems were more active (Pearson & Jakobsen, 1993).

Similarly, the results in the current study where shoot [P] increased up to 32% when inoculated with *R. irregularis* despite no increase in AMF-acquired P, suggests the direct root uptake pathway must be enhanced. It is conceivable that feedback mechanisms are at play to downregulate genes within the direct pathway when the hyphal contribution to P is high. On the other hand, AMF

species which contribute only small amounts of P may stimulate direct uptake pathways to ensure enough nutrient uptake to sustain photosynthesis and the supply of C to the fungal partner (Pearson & Jakobsen, 1993a). This hypothesis fits with the results of the current study where AMF supplied very low amounts of P to the host plants, and the root systems were supporting high levels of AMF colonisation, especially when inoculated with commercial *R. irregularis* inoculum.

Research has been conducted into P transporter genes within plants to explain these changes to the direct uptake pathway in response to associations with AMF. It has frequently been hypothesised that changes in expression of P transporter genes in the direct pathway lead to changes in direct P uptake, but this has seldom been shown (Grønlund et al., 2013). There is also evidence of functional diversity between AMF species in how they affect the expression of P transporter genes in the direct uptake pathway (Burleigh, 2002). Therefore, if community changes within the roots of wheat have taken place when inoculated with *R. irregularis*, this could explain changes to the direct uptake pathway and is an important area for further study (see Chapter 3).

Alternatively, the large increases in AMF colonisation seen in this study could explain the increase in the plant's direct uptake pathway through changes in stomatal conductance ( $g_s$ ) (Augé, 2001). A meta-analysis of 460 studies found that AMF symbiosis increased  $g_s$  by an average of 24% (Augé et al., 2014). Although the present experiment did not look at the difference between AM and non-AM plants, we did see a substantial increase in colonisation after the inoculation treatment, and the results of the meta-analysis indicate that heavily colonised plants could increase  $g_s$  ten times as much as lightly colonised plants. Increased transpiration has been shown to alter root architecture by increasing root hair length and density, consequently increasing nutrient uptake (Hepworth et al., 2016). This is a further mechanism by which inoculation with *R. irregularis* could have indirectly increased plant P uptake through the direct pathway.

The second hypothesis to explain the increase in plant [P] when plants were inoculated with *R. irregularis* despite no observed increase in mycorrhizal acquired  $^{33}\text{P}$ , is that the measure of mycorrhizal P uptake through the use of radio-labelled  $^{33}\text{P}$  was conducted only for the last ten days of the experiment. Total [P] in the plant tissue, which was increased when plants were inoculated with *R. irregularis*, quantifies P accumulation over the whole lifetime of the plant.

Therefore, the discrepancy between these two results could be explained by the AMF making the biggest contribution to plant P in the early stages of the plant's development, which were not measured during this experiment. Thus, it is important to adopt a lifetime fitness approach to symbiotic functioning, as judging the symbiosis at just one point in the plant lifetime may not accurately portray the true nature and functionality of the symbiosis (Lekberg & Koide, 2014a).

There were differences in the mycorrhizal acquired  $^{33}\text{P}$  depending on the wheat cultivar; cv. Skyfall generally received more  $^{33}\text{P}$  from an AMF partner than the other wheat cultivars. This suggests the wheat cultivars have genotype-specific responses to AMF. cv. Skyfall is the most recently developed variety out of those tested. Therefore, cv. Skyfall receiving the most AMF acquired P in this study disagrees with other studies which observed that modern crop cultivars (after 1950) were least responsive to AMF (Hetrick et al., 1993; Zhu et al., 2001; Zhang et al., 2019). Modern cultivars were predicted to be least responsive due to commercial breeding programmes focusing on selection for root systems that are highly effective at assimilating necessary nutrients from mineral fertilisers (Tawaraya, 2003). cv. Avalon and Cadenza both received very little P through AMF, therefore, despite the known differences in canopy architecture, root system size and disease resistance, responses to AMF were similar.

### **2.5.2.2 Nitrogen**

We observed no effect of inoculation with *R. irregularis* and subsequently increased root colonisation, on the concentration of N in the shoot tissue. This is despite observing changes in the amounts of  $^{15}\text{N}$  transferred to the host plants, suggesting that although AMF can transfer N to their host plants, the amount they transfer may not be significant enough in the context of the overall N status of the plant. Our results are in line with the long-held view that N uptake by plant hosts through their mycorrhizal partner is present but quantitatively insignificant, at least in comparison to P acquisition. Mycorrhizal symbiosis may be more beneficial for plant P uptake because inorganic N sources are far more mobile in the soil than P and consequently do not become depleted in the rhizosphere, meaning root access is not limited (Smith & Smith, 2011).

The literature estimates of the amounts of N transferred to the plant host can vary drastically. Some monoaxenic root organ culture studies have suggested that AMF could contribute between 20-50% of the root N (Toussaint et al., 2004;

Govindarajulu et al., 2005; Jin et al., 2005). However, in this study, we found a substantial increase in mycorrhizal colonisation created through the addition of *R. irregularis* in agricultural soil produced no increase in plant N assimilation. A possibility for the discrepancy between root organ culture studies and the current study is that high concentrations of inorganic N are added to the media in root organ cultures, whereas the plants in our study were in an environment where N was likely the limiting nutrient. When examining the plant N:P ratio's, values strongly suggested N deficiency, normal plant tissue N:P values are around 10:1 (mass basis) (Smith & Smith, 2011), whereas the current study values ranged from 3:1 to 6:1.

Although, the ecological significance of enhanced N acquisition through AMF associations will be greatest under conditions where N is limiting, AMF themselves have a high N demand and therefore will only supply N to the plant when their own demand is satisfied (Hodge & Fitter, 2010; Fitter et al., 2011). Therefore, the low [N] in the present study are likely to have resulted in competition between the AMF and plants for N resources and may have resulted in low transfer to the plant (Hodge & Fitter, 2010; Hodge & Storer, 2014).

Even though no gains in overall [N] were detected under the present experimental conditions, there was a considerable variation in the amount of mycorrhizal-acquired  $^{15}\text{N}$  depending on AMF treatment and wheat cultivar. This indicates AMF species–wheat cultivar specificity in the function of symbiosis with AMF. For example, cv. Skyfall  $^{15}\text{N}$  uptake was increased from 0 to 6.8  $\mu\text{g/g}$  when the plants were inoculated with *R. irregularis*. On the other hand, there was a significant decrease in mycorrhizal acquired  $^{15}\text{N}$ , from 4.1  $\mu\text{g/g}$  to 1.6  $\mu\text{g/g}$  when cv. Avalon was inoculated with *R. irregularis*, suggesting the different wheat cultivars may have genotype-specific responses to *R. irregularis*. Alternatively, the addition of *R. irregularis* could alter the AMF communities inside wheat cultivars differently, and this can only be confirmed through analysis of the AMF communities inside the roots. The negative response of cv. Avalon in terms of mycorrhizal acquired nutrients, when inoculated with *R. irregularis*, was also observed in the  $^{33}\text{P}$  acquisition. Wheat cultivars have been shown to have genotype-specific responses to AMF (Ocampo & Azcon, 1981; Hetrick et al., 1993; Zhu et al., 2001). Likewise, varying AMF communities within the plant roots may have influenced  $^{15}\text{N}$  uptake through functional diversity between different species or isolates of

AMF (Munkvold et al., 2004). One study, which measured the ability of 31 isolates of AMF to contribute to N nutrition of *Medicago sativa*, found a vast functional diversity within isolates, with high performing isolates able to increase [N] by 211% compared to non-mycorrhizal controls, whereas many isolates were unable to have any significant effect on tissue N (Mensah et al., 2015).

### **2.5.3 The effect of AMF inoculation on plant carbon resources allocated to the fungal partner and plant biomass.**

Although I noted a substantial increase in colonisation in the host plants when inoculated with *R. irregularis* (Figure 2.4), I did not see an increased allocation of C to the soil cores (Figure 2.8) or even increased [C] in the roots which could have been allocated to the intra-radical mycelium (Figure 2.7). C allocation to extra-radical mycelium was calculated to be no more than 2% of recently-fixed plant C, which is lower than previous estimates that 4-20% of plant photosynthate is allocated to supporting a fungal partner (Smith & Read, 2008). However, estimates equivalent to in this experiment have also been found, for example, <sup>13</sup>C-labelling of *Plantago lanceolata* associated with a *G. mosseae* (now *Funneliformis mosseae*) found C demand of the extraradical mycelium was less than 1% of net photosynthesis (Heinemeyer et al., 2006). Recent evidence suggests plant C allocation to AMF may be lower than previously estimated and frequently falls below 10% of the plant C budget (Konvalinková et al., 2017).

The results of the present experiment suggest wheat gains little P or N through its mycorrhizal partner and provides little C in return. The seemingly low resource exchange within the symbiosis could be explained if the mechanism of resource exchange is regulated by direct stimulation of C release within the plant root in response to P release by AMF across the periarbuscular membrane (Fitter, 2006). This hypothesis is based on the known root response of allocating resources to nutrient rich zones, e.g. through the proliferation of lateral roots (Helgason & Fitter, 2009; Fitter et al., 2011). Therefore, when plants detect a heightened concentration of nutrients released via AMF structures, they will respond by enhancing C supply to this area which can be acquired by the AMF (Helgason & Fitter, 2009; Fitter et al., 2011). Within this experiment, there was no detected increase in mycorrhizal acquired P after inoculation, this theory could explain the lack of increase in C flow below ground despite the large increase in colonisation.

However, the significant increase in root colonisation in plants inoculated with *R. irregularis* could be suggestive of higher C allocation earlier in the symbiosis. As the wheat plant is the AMF's only C source, they must have gained adequate resources from the plant to increase investment in fungal structures. Therefore, C-for-P exchange may have been greater earlier on in the plant lifecycle (Section: 2.5.2.1). Alternatively, even though C allocation to the fungus is a small proportion of the plant's C budget (Figure 2.8c), it may not be limiting fungal growth. If this is the case, the limitations on AMF colonisation in the non-inoculated plants may have been low spore densities in the agricultural soil only treatment (Lekberg & Koide, 2005).

Overall, there is little evidence from the present experiments that AMF are acting as a significant C drain on wheat C resources across all cultivars tested which could otherwise have been used for enhancing plant growth and fitness. Although, there were differences in the amount of C allocated to the fungi between wheat cultivars in this study, with cv. Avalon allocating the most C to its fungal partner. Higher C transfer within cv. Avalon could be due to a different community composition within roots with a higher C demand (Pearson & Jakobsen, 1993a), although this would need to be confirmed through community analysis.

Due to the negligible C drain on the plant and the increase in plant [P] (either through stimulation of the plant direct uptake pathway or mycorrhizal uptake earlier in the plant lifecycle), it is perhaps surprising that I found no increase in shoot or root biomass in any of the wheat cultivars tested. In cv. Avalon and cv. Cadenza, root biomass significantly decreased. This decrease in root biomass represents a C saving that was not, as far as it possible to see, transferred to the AMF.

There are many examples in the literature of plants which are unresponsive to AMF colonisation in term of biomass changes (Reynolds et al., 2005; Chu et al., 2013), even in soil of low P status and even in cases where [P] within the plant increase (Wilson & Hartnett, 1998; Tawaraya, 2003; Klironomos, 2003). One possibility for the lack of growth response, despite increases in tissue P, is that the plants were N rather than P limited. This is supported by the plants low N:P ratio and overall low tissue [N]. However, if inoculation of agricultural soil with AMF can make a significant contribution to plant P at no extra C cost, it could

result in positive plant growth responses under conditions where P and not N is limiting plant growth.



## 2.6 Conclusions

This study has reaffirmed the complexity of the symbioses between AMF and their plant hosts. Despite concerns that commercial AMF inoculants might not be able to persist in new environments with already present AMF communities, the *R. irregularis* inoculant in this study was able to successfully increase the colonisation of all wheat hosts, independent of the cultivar. This reaffirms that the inoculum potential of agricultural soil is low and limits crop colonisation, supporting my first hypothesis.

This experiment also contributes evidence that the use of inoculants may enhance plant P uptake, and therefore inoculation with such products could have the potential to decrease the reliance on phosphate fertilisers in the future. Again, this was independent of the cultivar tested. However, we were unable to attribute this increased P in the plant tissue directly to P uptake through the mycorrhizal pathway using radio-labelled  $^{33}\text{P}$ , leaving the mechanism behind the increase in plant P unknown.

This experiment has additionally contributed to the evidence that AMF can pass inorganic N to their plant partner, although the amounts may be inconsequential in terms of total plant N. Cultivar differences in mycorrhizal-acquired  $^{15}\text{N}$  in response to inoculation with *R. irregularis* were apparent. However, although we saw a significant increase in the amount of mycorrhizal acquired  $^{15}\text{N}$  in cv. Skyfall and a significant decrease in cv. Avalon, these results did not affect tissue N values. These cultivar specific responses in  $^{15}\text{N}$  acquisition, provide evidence for a cultivar-AMF species-specific response. cv. Avalon, in particular, responded negatively, with a significant decrease in both mycorrhizal  $^{33}\text{P}$  and  $^{15}\text{N}$  when inoculated with *R. irregularis*. These cultivar differences have often been observed, but the mechanism remains unknown and is essential to consider before use of AM inoculants are encouraged in agricultural systems where the crop cultivars that are grown can change frequently. The cultivar differences could be explained by a cultivar specific response to *R. irregularis*, and important next steps include confirming through community analysis that *R. irregularis* was the main coloniser after inoculation.

C allocation from the plant to their mycorrhizal partner was assessed using  $^{14}\text{CO}_2$ , and despite the increase in root colonisation in inoculated plants, we found no evidence of increased C drain on the plants.

Overall, we found adding *R. irregularis* inoculant to an agricultural soil did not increase shoot biomass of the plants. However, the increase in plant P for no apparent C 'cost' to the plant is promising in terms of reducing potential future reliance on P-based fertilisers, and under different nutrient regimes, where P was the limiting growth factor, may have contributed to increased growth and yield of the wheat plants.

## **Chapter 3 The effect of *Rhizophagus irregularis* inoculation on the intra-radical community assembly of three wheat cultivars.**

### **3.1 Introduction**

Conventional farming practices such as over-usage of fertilisers, tillage, long fallow periods and crop rotations decimate AMF diversity in agricultural soils (Helgason et al., 1998; Daniell et al., 2001). These practices disturb extraradical hyphal network development, creating long periods where AMF have limited access to host plants and thus, a C supply (Helgason et al., 1998; Jansa et al., 2002). AMF diversity and abundance in agricultural soils could be improved by changing management practices to promote native AMF abundance, or by reintroducing AM fungal spores and propagules through inoculation (Lekberg and Koide 2005).

Commercial AMF products are readily available and aim to boost AMF spore density in soils, acting as 'biofertilisers', allowing crops to effectively utilise existing soil N and P pools (Vosátka et al., 2012; Faye et al., 2013). The use of AMF inoculants in arable fields relies on inoculant additions being economically viable and easy to apply (see section 1.1.8); and inoculation with AMF has been successful in some cases (Pellegrino et al., 2015; Hijri, 2016). Additionally, with new technology such as seed coating, which requires much smaller volumes of inoculum and no new farming equipment (Oliveira et al., 2016), field-scale inoculation is perhaps becoming a more realistic prospect. However, after the addition of AMF inoculum to soil, little is known about what happens to the AMF community composition and structure within plant roots. AMF species undergo complex interactions, including both facilitation and competition (Callaway & Walker, 1997). From an ecological perspective, there is some concern that AMF inoculants, usually containing a single generalist species (commonly *Rhizophagus irregularis*) selected to be a competitive coloniser with a high sporulation rate, could become invasive and suppress native AMF communities (Schwartz et al., 2006; Antunes et al., 2009; Hart et al., 2017). Traits commonly associated with invasive species include having a broad host range, a high competitive ability compared to native species, and high dispersal rates (Schwartz et al., 2006).

In some cases, native AMF species appear to be more beneficial for their host plants than species introduced via inoculation. For example, inoculants containing native AMF species produced higher cassava yields (Séry et al., 2016), enhanced salinity tolerance in maize (Estrada et al., 2013), and produced higher biomass and herbivory tolerance at prairie restoration sites (Middleton et al., 2015) than counterparts introduced as commercial inoculants. Consequently, if commercial strains of AMF outcompete native AMF species and reduce plant root colonisation, they could unintentionally reduce AMF-acquired benefits to the plant. However, there are examples where a commercially available AMF inoculum increases plant productivity (Ceballos et al., 2013; Hijri, 2016), so results are not consistent and may represent general variation in effectiveness between the AMF species tested. For example, there is a range of parasitic to mutualistic responses when using both native and foreign fungal genotypes (Klironomos, 2003). Although, the relative frequency of positive plant growth responses was found to decrease with foreign AMF genotypes (Klironomos, 2003).

It has become apparent in the last 30 years that symbiotic outcomes are variable between different species, even between genotypes of plants and isolates of AMF (Tawaraya, 2003; Munkvold et al., 2004; Hoeksema et al., 2010). Studies in cucumber (*Cucumis sativus*) (Munkvold et al., 2004) and alfalfa (*Medicago sativa*) (Mensah et al., 2015), have shown that variation in plant growth responses can be higher when inoculated with different isolates of AMF rather than with diverse species. Intra-specific variation could not be associated with fungal morphology or growth, which is typically highly conserved within species (Koch et al., 2017). There was also no consistency in the effects of AMF isolates on different plant hosts, showing plant species × AMF intraspecific isolate interactions.

In some cases, AMF can acquire large amounts of plant photosynthates and may represent a drain on plant's C resources (Smith & Read, 2008). This can result in negative plant growth responses if fungal demands for C outweigh any nutritional or non-nutritional benefits provided to the plant host. If AMF strains within commercial inoculants are not mutualistic for the host plant, vastly increasing abundance in the soil could exacerbate any C drain on the host with no perceived benefit. However, evidence presented in the previous chapter

suggests substantial increases in root colonisation can be achieved after inoculation without a significant increase in C investment by the plant. This indicates plant C investment may not be limiting fungal abundance under the conditions of this experiment. I have also shown that the addition of a commercial AMF inoculum to agricultural soil can have varying outcomes across wheat cultivars (Chapter 2). However, I did not determine if different communities of native fungi colonised the plant roots to explain these cultivar differences.

Many factors could determine the intra-radical community structure within plant roots, for example competitive interactions among AMF species are predicted to be a vital driver community composition (Werner & Kiers, 2015). When three AMF species (*Glomus mosseae*, *G. claroideum* and *G. intraradices*, now *Funneliformis mosseae*, *Claroideoglomus claroideum* and *Rhizophagus irregularis* respectively) were inoculated singly or in combination in the roots of *Medicago* or *Allium*, *F. mosseae* was always the best competitor in terms of highest fungal abundance in the roots (Jansa et al., 2008). Fungal abundance was measured using real-time PCR to determine ribosomal large subunit (LSU) copy number, and co-inoculation with *F. mosseae* consistently reduced the LSU copy numbers of the co-colonising fungi. Agricultural soils are often found to be rich in *Glomeraceae* species, including *F. mosseae* (Daniell et al., 2001; Jansa et al., 2002; Oehl et al., 2003; Rosendahl, 2008), which may reduce the host plant colonisation by a commercial inoculant containing less competitive AMF species. Mechanisms for the competitive dominance of *F. mosseae* could include different modes of root colonisation between the fungal species; for example, *F. mosseae* was the fastest coloniser (Jansa et al., 2008). The time it takes for certain fungal species to colonise the roots can vary significantly (Hart & Reader, 2002), the fastest colonisers may have a superior ability to colonise the roots through means other than spores, such as fungal mycelium and infected root fragments.

The order of arrival of AMF species to the plant root system is a central factor which determines colonisation success and subsequent community development (Verbruggen et al., 2013). However, there is also evidence for succession in AMF communities and changes across the growing season (Sýkorová et al., 2007; Bennett et al., 2013). Therefore, the community composition measured at any one plant life-stage may not be representative of all stages, especially when

plants are grown outside of controlled conditions, as varying environmental conditions could favour different fungal partners.

When the first species to arrive at a site has a substantial effect on the ensuing community structure, the effects are defined as 'priority effects'. Priority effects can be inhibitory to future arrivals if they reduce the availability of space and resources for subsequent colonisers, or they may be facilitative if they alter conditions in a favourable way for later arrivals (Fukami, 2015). Priority effects could affect competition between an AMF inoculant and native species (Mummey et al., 2009; Werner & Kiers, 2015). Under semi-controlled greenhouse conditions, Werner and Kiers (2015) showed the second mycorrhizal fungus to arrive at a plant root had a reduced abundance in both species tested (*Glomus aggregatum* and *R. irregularis*). This suppression was increased the longer the 'head start' of the first colonising fungus, with a significant reduction of 97% after four weeks. While the mechanism behind the suppression is not fully understood, a hypothesis is that competition for host root space is significant (Mummey et al., 2009). Alternatively, there is evidence that colonisation by AMF may negatively regulate further colonisation (Vierheilig et al., 2000; Vierheilig, 2004) through downregulation of strigolactone production (López-Ráez et al., 2011). Strigolactones are exuded by plant roots into the rhizosphere and allow AMF to detect host plants and trigger hyphal branching (Akiyama et al., 2005). One recently proposed model for plant regulation of colonisation is that a feedback loop occurs upon colonisation, triggering higher expression of CLAVATA3/ESR-related (CLE) peptides which have been associated with modulating colonisation via suppressing strigolactone biosynthetic gene expression (Müller et al., 2019).

Consequently, if the native AMF community has an established hyphal network in the soil, its initial colonisation of the plant may radically reduce the effectiveness of any inoculant additions at colonising the host roots. Although, in an agricultural setting native AMF populations tend to be low (Oehl et al., 2003), and hyphal networks are often disturbed by tillage before planting, so inoculant additions may be successful as the native AMF species would not have an established hyphal network in place (Verbruggen et al., 2013). There is growing evidence that inoculum additions can successfully increase the AMF populations in the soil and the colonisation of host plant roots (Lekberg & Koide, 2005; Pellegrino & Bedini, 2014; Köhl et al., 2016). This includes data presented in the

previous chapter, which showed *R. irregularis* inoculum was able to successfully increase the colonisation of three wheat cultivars grown in the presence of a native AMF community.

There is a lack of evidence regarding the effect of inoculum additions on the community composition of AMF in plant roots. This is a critical knowledge gap as AMF inoculants containing highly competitive species may lead to a decrease in AMF diversity or richness, which could result in a decline in AMF-acquired benefits, due to evidence of functional diversity between AMF species (Jansa et al., 2008; Antunes et al., 2009; Verbruggen et al., 2012). Alternatively, there is evidence that some commercial inoculants may fail to establish altogether and therefore, confirming commercial inoculants colonise plant roots by community analysis is essential.

In the previous chapter, the addition of a *R. irregularis* commercial inoculant resulted in a significant increase in root colonisation. However, the increase in colonisation varied widely between the wheat cultivars suggesting differences in the ability of *R. irregularis* to colonise the different wheat cultivars. Variation in the extent of AMF colonisation may be due to cultivar-AMF specificity. However, it is important not to presume the increase in root colonisation is exclusively *R. irregularis*. Interactions such as facilitation and competition could result in native AMF species having an increased presence in inoculated plants.

Carbon-for-nutrient exchange within the symbiosis was also affected by the addition of inoculum, particularly in regards to  $^{15}\text{N}$  uptake. For example, cv. Skyfall  $^{15}\text{N}$  uptake was increased from 0 to 6.8  $\mu\text{g/g}$  when the plants were inoculated with *R. irregularis* compared to native-AMF only treatments. On the other hand, there was a significant decrease in mycorrhizal acquired  $^{15}\text{N}$ , from 4.1  $\mu\text{g/g}$  to 1.6  $\mu\text{g/g}$  when cv. Avalon was inoculated with *R. irregularis*. However, without community analysis, these observations could not be assigned as a response to *R. irregularis*. The increase in root colonisation in the inoculated plants could be exclusively *R. irregularis* or an increased abundance of mixed native AMF species. If only *R. irregularis* abundance increased after inoculant additions, cultivar differences could be explained by a cultivar-specific response to *R. irregularis*. Alternatively, if changes in AMF community composition are different between the wheat cultivars, differences in carbon-for-nutrient exchange could be explained by varying AMF community compositions. For example,

variation in AMF communities within the plant roots could have influenced  $^{15}\text{N}$  uptake through functional diversity between different species.



### 3.2 Aims and Hypotheses

Here, I address the key questions arising from Chapter 2. Specifically:

- Is the higher root colonisation in the inoculated plants exclusively *R. irregularis* from the inoculum added, or does it reflect increased abundance of mixed native AMF species?
- Did addition of commercial *R. irregularis* inoculum change the AMF community structure or diversity within the plant roots?
- Can cultivar differences in carbon-for-nutrient exchange be explained by differences in the AMF community compositions or a cultivar-specific responses *R. irregularis*?

The objective of this study was to answer these questions by quantifying how application of commercial *R. irregularis* inoculum changed the AMF community composition and structure in three wheat cultivars using T-RFLP.

I test the following hypotheses:

1. Addition of *R. irregularis* inoculum to the soil will cause changes to the mycorrhizal community colonising plant roots. *R. irregularis* will dominate the colonisation of the plant roots due to the high density of inoculum close to the root system, meaning *R. irregularis* will likely be the first coloniser and gain an advantage through access to the plants carbon resources. This has been shown previously to reduce the abundance of subsequent colonisers, in this case, the native AMF species.
2. Wheat will have cultivar-specific responses to inoculum additions. Cultivar-specific responses may manifest as differences in root length colonised by AMF after inoculation and differences in the AMF communities colonising the roots. This hypothesis is based on cultivar differences seen in responses to inoculation in the previous chapter. Specifically, cultivars which had the smallest change in colonisation after inoculation (cv. Skyfall) may be expected to have a less altered community. cv. Avalon, which had the largest increase in colonisation and exhibited a significant decrease in AMF acquired P and N after inoculation, may have had the most drastic community change to a less favourable symbiont.

### **3.3 Materials and Methods**

#### **3.3.1 Biological material and growth conditions**

Wheat seeds (*Triticum aestivum* L. – cvs. Cadenza, Skyfall, Avalon) were sterilised with chlorine gas, germinated on damp filter paper at 20°C for six days then transplanted into pots containing agricultural soil and sand in a 1:1 mix. Plants were grown in Jumbo root trainer pots (Haxnicks Tildenet) (24cm x 6.5 cm x 7 cm). Agricultural soil was collected from Leeds University Farm in May 2017 (Spen Common Lane, Tadcaster, North Yorkshire, LS24 9NU, England). The soil, a slightly alkaline sandy clay loam, was air-dried overnight in trays and passed through a 2 mm sieve. The soil was stored at room temperature for seven days until planting. At the time of planting the wheat, seedlings were inoculated with a commercially available inoculum (20 g) advertised to contain a single species *R. irregularis* (PlantWorks, Kent, UK) or a sterilised control with the same carrying substrate. Therefore, the six treatments tested consisted of three wheat cultivars grown in non-sterile soil with *R. irregularis* commercial inoculum or non-sterile soil without active *R. irregularis* commercial inoculum, each treatment contained 8 replicates. The inoculum contained a mixture of infective AMF propagules including colonised root fragments, hyphae and spores and a carrying substrate (1:1 pumice and zeolite). The plants were grown in greenhouse conditions which were supplemented with LED lighting and electronic blinds to create a 16-hour photoperiod, light intensity: 350  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The plants were watered every three days, with each being given 30 ml of 40% nitrate type Long Ashton solution weekly (Hewitt, 1966).

#### **3.3.2 Plant harvest and tissue analysis**

Plants were grown as described above and harvested after eight weeks. The shoot and root biomass were separated, and soil was carefully washed from the roots with water. A sample of the roots was removed for colonisation analysis and the remaining plant material was freeze dried and homogenised before further analysis. The effect of AMF inoculation was measured on plant biomass, plant tissue [P] and percentage root length colonised with AMF, using methods as described previously (See section 2.3).

### **3.3.3 AM fungal PCR and T-RFLP**

#### **3.3.3.1 DNA extraction from plant roots**

The total DNA was extracted from between 10-20 mg of freeze-dried root material, which was placed in a safe-lock 2 ml Eppendorf tube with a 5 mm stainless steel bead (Qiagen, Hilden, Germany) and homogenised using a TissueLyser LT (Qiagen, Hilden, Germany) at 50 Hz for 1 minute (or until root tissue is ground to a fine powder). Fungal DNA was extracted using the protocol provided by the Plant DNeasy mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The DNA elution was tested for the quality and quantity of DNA on a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, Massachusetts, USA). Samples which exhibited low quality or quantity of DNA were repeated. Extracted DNA was stored at -20 °C.

#### **3.3.3.2 PCR**

A region of the small subunit rRNA (approximately 560 bp) was amplified using the general eukaryotic forward primer NS31(5'-TTG GAG GGC AAG TCT GGT GCC-3') labelled with a FAM fluorescent marker at the 5' end (Simon et al., 1992), and AMF specific reverse primer AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C - 3') (Lee et al., 2008). The PCR was carried out in a 20 µl reaction using 2 µl of DNA template, 10 µl of Qiagen Mastermix (Qiagen, Hilden, Germany) and 0.5 µM of each primer, made up to the final volume with PCR water. Thermal cycling consisted of an initial DNA denaturation step of 3 min at 94°C followed by 35 cycles each of 30 s at 94°C, 40 s at 59°C and 60 s at 72°C with a final extension step of 10 min at 72°C, on a 96-well thermal cycler. All PCR plates included a negative control to ensure that no DNA contamination was present. Gel electrophoresis was used to verify the success of PCR amplification. 1 µl of loading buffer (Bioline, London, UK) and 4 µl of PCR product was run on a 1.5% agarose gel with 0.001 % (v/v) SYBR® Safe DNA stain (Invitrogen, Carlsbad, USA). PCR products were run along-side a 100 bp ladder (Bioline, London, UK).

#### **3.3.3.3 Restriction enzyme digests**

PCR products were digested in two separate digests with the restriction enzymes HpyCHIV, MboII and Sau96I (New England Biolabs, Inc) in one digest and Bfal and Bsrl (New England Biolabs, Inc) in another digest. Previous work showed these combinations to be most discriminatory of AMF genotypes found in

agricultural field soil (van den Bos, 2015). For the HpyCHIV/MboII/Sau96I digest, the optimal reaction set-up contained 1 unit of each enzyme, 1 µl of CutSmart buffer (All enzymes had 100% activity in CutSmart buffer) and 3 µl of PCR product, made up to final reaction volume of 10 µl with water. Digests were incubated for 60 minutes at 37°C then denatured at 65°C for 20 minutes. For the Bfal/Bsrl digest, there was no buffer in which both Bfal and Bsrl exhibit >50% activity due to differences in optimal salt concentrations, thus a sequential digest was necessary. The initial reaction set-up contained 1 unit of Bfal enzyme, 1 µl of CutSmart buffer and 3 µl of PCR product, made up to final reaction volume of 10 µl with water. Reactions were incubated at 37°C for 60 minutes and denatured by incubating at 80°C for 20 minutes. To bring the salt concentration up to 100 mM, optimal for the second enzyme, 0.5 µl of a 2 M NaCl solution was added to the initial reaction, along with 1 unit of Bsrl (20% v/v mixture with NEBuffer 3.1). The reaction was incubated for a further 60 minutes at 65°C and denatured by incubating at 80°C for 20 minutes. All digests were run alongside uncut samples and negative controls.

Digests were diluted 1:10 with water to prepare the samples for capillary DNA analysis. 1 µl of the diluted digest was added to 9 µl of formamide containing 1% GeneScan™ LIZ 1200 size standard (Applied Biosystems, UK) and heated at 94°C for 3 minutes before immediate cooling on ice. Genotyping was carried out on an ABI 3730 PRISM® capillary DNA analyser (Applied Biosystems, UK).

### **3.3.4 T-RFLP data processing and analysis**

T-RFLP data was analysed using Genemapper software v. 5 (Applied Biosystems, UK), with a background threshold of 50 fluorescent units, and a bin width of 5 bp. Peaks were analysed in the range of 50-850 bp. The relative abundance of each peak was calculated to the percentage of total sample fluorescence. Peaks containing <1% of the total sample fluorescence were discarded, and artefacts were detected by identifying peaks which frequently occurred in both the cut and uncut samples, after their removal the proportion of total sample fluorescence accounted for by the peaks was recalculated. Samples of the commercial inoculum were also analysed by T-RFLP to assess community composition within the inoculum which was advertised to contain only *R. irregularis*. The SSU sequences of AMF species commonly associated with agricultural soils were downloaded from GenBank and virtually digested with

RestrictionMapper v. 3, to associate T-RF's with potential AMF species. Examination of the Bfal/Bsrl digest T-RFs, compared with those expected in virtual digests suggested that this digest reaction was incomplete, and many products remained only partly digested, making community analysis less robust.

The incomplete digestion of the Bfal/Bsrl digest T-RFs was likely due to differences in the enzymes optimal salt concentrations, meaning a sequential digest was necessary to try and bring the enzymes up to optimal efficiency. However, this methodology was not successful; therefore, only the results of the HpyCHIV/MbolI/Sau96I digest will be discussed.

### **3.3.5 Statistical analysis**

Analysis of root colonisation, plant tissue P content and plant biomass, between plant grown in non-sterile soil + *R. irregularis* and non-sterile soil – *R. irregularis* and whether impact differed between three wheat cultivars was assessed by two-way ANOVA followed by TukeyHSD tests using Minitab (Version 17). Before statistical analysis, data were checked for confirmation to assumptions of normality and equal variance using normal probability plots and residuals vs fits plots. In the case of % vesicles, the data were transformed using the optimal lambda function in Minitab (Version 17), to fulfil data assumptions.

AMF community fingerprint data collected from T-RFLP analysis was analysed using the Vegan: Community Ecology Package (R package version 2.5-6). The number of T-RFs per sample (T-RF richness) and the Shannon diversity index of T-RF's was calculated, and differences between inoculated and non-inoculated plants and differences between wheat cultivars were analysed by a two-way ANOVA using Linear Models. The data were checked for confirmation to assumptions of normality and equal variance using Normal Q-Q and Residuals vs Fitted plots. The impact of inoculation on community composition and structure was analysed by Principal Component Analysis (PCA) using the Vegan: Community Ecology Package (R package version 2.5-6). PCA was performed on relative abundance data using the function 'rda'. PCA scores were generated for the first 4 axes, and the proportion of variation captured by these axes was visualised using scree plots. The effect of inoculation treatment and wheat cultivar on the PCA scores was assessed using two-way ANOVA. T-RF loading scores were generated by the analysis and used to infer to what extent different

T-RF peaks were driving the separation of the communities on the ordination graphs.

Lastly, a permutational multivariate analysis of variance (PERMANOVA) was conducted using the 'adonis' function in vegan, to assess if communities differed significantly between inoculated and uninoculated plants or different wheat cultivars. The homogeneity of group dispersions assumption was assessed by the 'betadisper' function, which is a multivariate equivalent of Levene's test for homogeneity of variances. Differences were evaluated visually by boxplots and by ANOVA, and no significant differences in dispersion were detected.

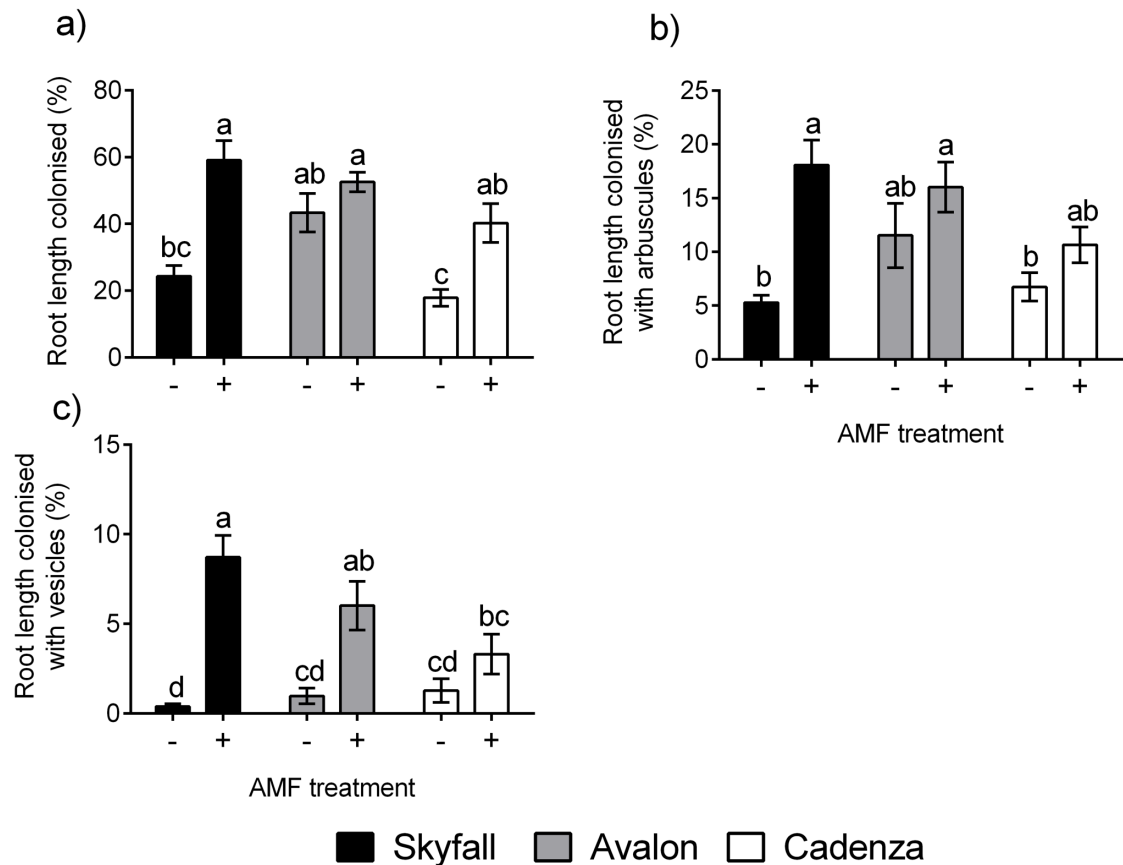
### 3.4 Results

#### 3.4.1 Root colonisation

In non-inoculated plants root length colonisation by AMF varied between a mean of 24%, 43% and 18% in cv. Skyfall, Avalon and Cadenza respectively when grown in the presence of only the native AMF community. Supplementing agricultural soil with commercial inoculum resulted in higher mycorrhizal colonisation within the roots of wheat plants. However, the extent of the increases in colonisation varied between wheat cultivars (Interaction:  $F=3.9$ ,  $d.f.=2,42$ ,  $P=0.029$ ; two-way ANOVA), with only cv. Cadenza and cv. Skyfall increasing significantly (Tukey  $P<0.05$ ). Inoculated plants had an average root length colonisation of 59%, 52% and 40% in cv. Skyfall, Avalon and Cadenza, respectively. AMF structures such as arbuscules and vesicles were also more abundant within the root system of inoculated wheat plants compared to non-inoculated plants overall (arbuscules:  $F=18.2$ ,  $d.f.=1,42$ ,  $P<0.001$ ; two-way ANOVA), although the increase in vesicle structures up on inoculation varied between the wheat cultivars (vesicles: interaction  $F=5.3$ ,  $d.f.=2,42$ ,  $P=0.009$ ; two-way ANOVA; Figure 3.1b,c).

**Table 3.1:** Summary of two-way ANOVA (GLM) results testing the effects of inoculation with *R. irregularis* on root colonisation in three wheat cultivars. Bold indicates significant differences, where interactions were significant main effects were not reported.

<b>Variable</b>	<b>AMF treatment</b>		<b>Wheat cultivar</b>		<b>AMF Cultivar x</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	<i>1, 42</i>	<i>d.f</i>	<i>2,42</i>	<i>d.f</i>	<i>2,42</i>
Root colonisation (%)	34.7	<0.001	8.8	0.001	3.9	<b>0.029</b>
Arbuscules (%)	18.2	<b>&lt;0.001</b>	3.2	0.052	3.0	0.060
Vesicles (%)	58.2	<0.001	1.6	0.214	5.3	<b>0.009</b>

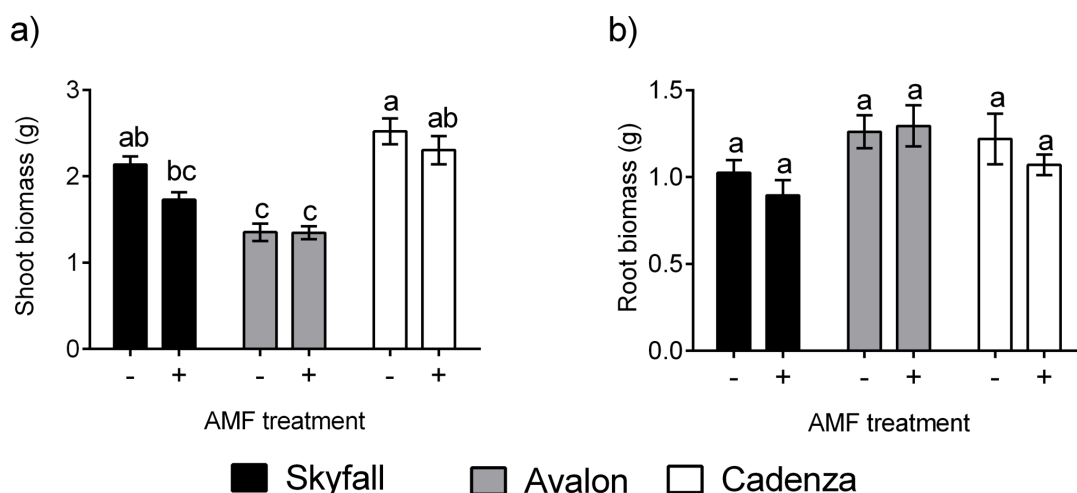


**Figure 3.1: Percentage of the root length colonised by arbuscular mycorrhizal fungi with or without inoculation with *R. irregularis*.** (a) total colonisation, (b) arbuscules, (c) vesicles. Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and Cadenza (white bars). Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Error bars represent the standard error of the mean. Different letters refer to significant differences (n=8,  $P<0.05$ , two-way ANOVA, TukeyHSD post-test) between treatments.

### 3.4.2 Plant biomass.

Although the use of a AMF inoculum in agricultural soil had no impact on shoot biomass within each wheat cultivar (TukeyHSD,  $P>0.05$ ), the overall effect of adding commercial inoculum was negative ( $F=6.23$ , d.f.=1,42,  $P=0.017$ ; two-way ANOVA; Figure 3.2a, Table 3.2). The addition of a commercial inoculant to agricultural soil did not affect root biomass in any of the cultivars tested ( $F=0.05$ , d.f.=1,42,  $P=0.825$ ; two-way ANOVA; Figure 3.2b, Table 3.2).





**Figure 3.2: Wheat plant biomass with or without inoculation with *R. irregularis*** (a) shoot biomass (b) root tissue biomass. Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and Cadenza (white bars). Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Error bars represent the standard error of the mean. Different letters refer to significant differences between treatments (n=8,  $P < 0.05$ , two-way ANOVA, TukeyHSD post-test).

**Table 3.2:** Summary of two-way ANOVA (GLM) results testing the effects of inoculation with *R. irregularis* on biomass in three wheat cultivars. Bold indicates significant differences.

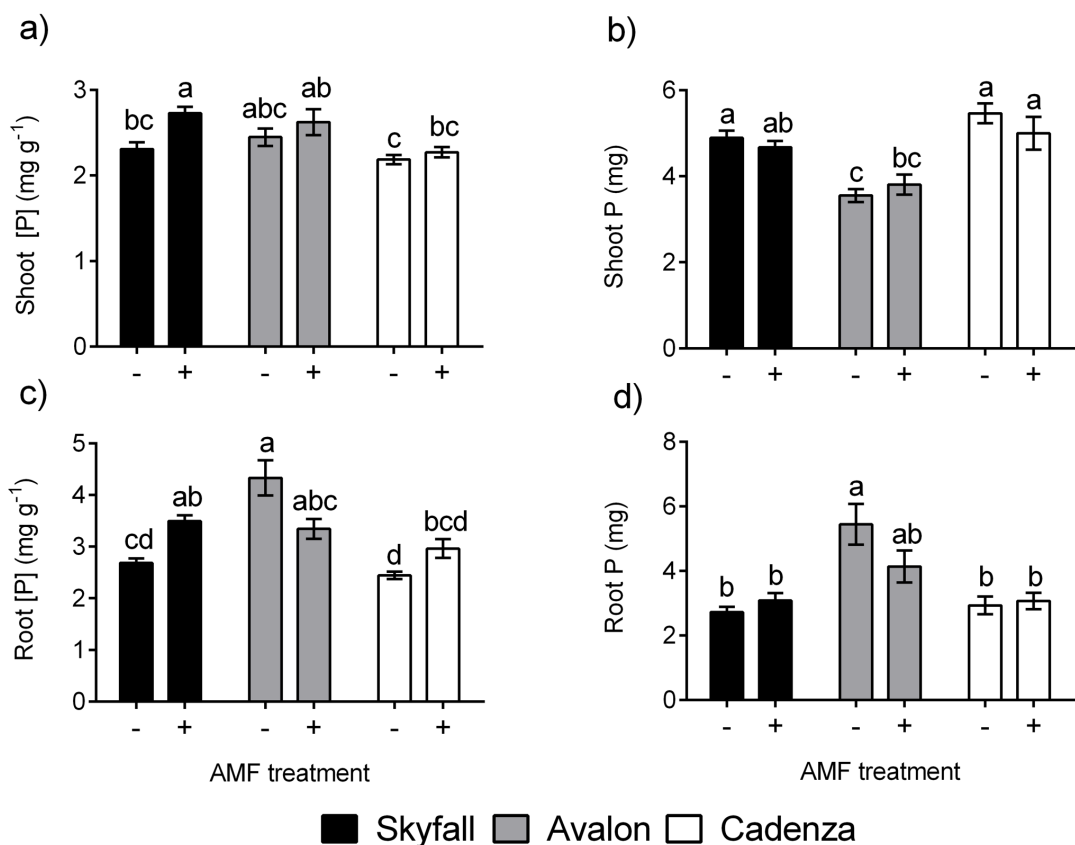
<b>Variable</b>	<b>AMF treatment</b>		<b>Wheat cultivar</b>		<b>AMF Cultivar</b>		<b>x</b>
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	
Degrees of freedom	d.f	1, 42	d.f	2, 42	d.f	2, 42	
Shoot biomass (g)	16.3	<b>0.017</b>	36.4	<b>&lt;0.001</b>	1.6	0.221	
Root biomass (g)	0.05	0.531	2.92	0.065	0.6	0.531	

### 3.4.3 Plant tissue phosphorus.

Adding *R. irregularis* inoculum increased shoot tissue [P] slightly in all wheat cultivars compared to the control plants in agricultural soil only ( $F=9.73$ , d.f.=1,42,  $P=0.003$ ; two-way ANOVA; Figure 3.3a). However, total amounts of P in the

shoot material were not affected by inoculation with *R. irregularis* ( $F=0.57$ ,  $d.f.=1,42$ ,  $P=0.456$ ; two-way ANOVA; Figure 3.3b).

Changes in [P] in the root was dependent on wheat cultivar (Interaction:  $F=12.1$ ,  $d.f.=1,42$ ,  $P<0.001$ ; two-way ANOVA; Figure 3.3c). In cv. Skyfall and cv. Cadenza, there was an increase in [P] in the root tissue, but no increase in overall P accumulation. In contrast, there was a decrease in [P] within the root system in cv. Avalon, along with no change in total P ( $F=0.10$ ,  $d.f.=1,42$ ,  $P=0.75$ ; two-way ANOVA; Figure 3.3d). These results vary from the previous chapter findings where the inoculum additions resulted in a significant increase in P uptake and concentration within the wheat cultivars.



**Figure 3.3: Phosphorus assimilation by the host plant with or without inoculation with *R. irregularis*.** (a) Plant shoot tissue concentrations of phosphorus ([P]), (b) total plant shoot tissue P, (c) plant root tissue [P], (d) total plant root tissue P. Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and Cadenza (white bars). Error bars represent the standard error of the mean. Different letters refer to significant differences between treatments. ( $n=8$ ,  $P<0.05$ , two-way ANOVA, TukeyHSD post-test).

**Table 3.3:** Summary of two-way ANOVA (GLM) results testing the effects of inoculation with *R. irregularis* on phosphorus assimilation in three wheat cultivars. Bold indicates significant differences, where interactions were significant main effects were not reported.

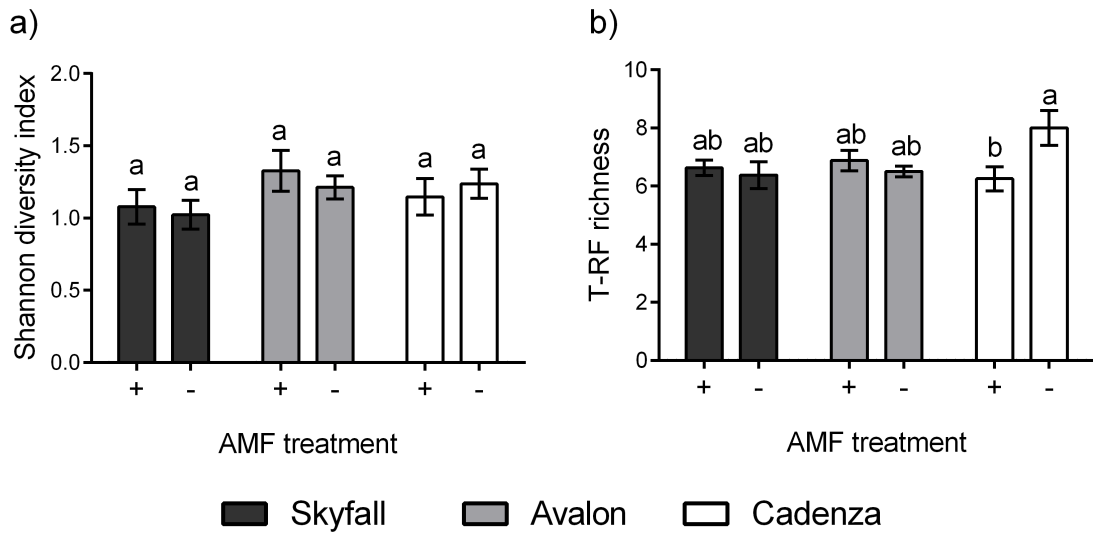
<b>Variable</b>	<b>AMF treatment</b>		<b>Wheat cultivar</b>		<b>AMF x Cultivar</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	1, 42	<i>d.f</i>	2,42	<i>d.f</i>	2,42
Shoot [P] (mg g <sup>-1</sup> )	9.7	<b>0.003</b>	7.6	<b>0.002</b>	2.0	0.153
Shoot P (mg)	0.6	0.456	23.6	<b>&lt;0.001</b>	1.2	0.299
Root [P] (mg g <sup>-1</sup> )	3.2	0.081	19.2	<0.001	12.1	<b>&lt;0.001</b>
Root P (mg)	0.1	0.750	13.9	<b>&lt;0.001</b>	2.3	0.113

#### 3.4.4 T-RFLP community analysis.

A total number of 18 T-RFs were observed, and T-RF richness (the number of T-RFs in a particular plant root system) was generally consistent between the inoculated and uninoculated treatments, apart from in cv. Cadenza where it decreased in the plants grown in soil inoculated with *R. irregularis* (Interaction:  $F=4.4$ ,  $d.f.=2,42$ ,  $P=0.018$ ; two-way ANOVA; Figure 3.4b). The Shannon diversity index of the T-RFs remained unaffected by either inoculation treatment or wheat cultivar (AMF treatment:  $F=0.1$ ,  $d.f.=1,42$ ,  $P=0.775$ ; Wheat cultivar:  $F=1.9$ ,  $d.f.=2,42$ ,  $P=0.156$ ; two-way ANOVA; Figure 3.4a). The similarity of T-RF profiles was used to assess community composition. Despite the similarities in richness and diversity, the community composition within the roots was significantly changed between plants grown in the presence of the native AMF community only and those grown in the native community and inoculant treatment ( $F=60.0$ ,  $d.f.=1,42$ ,  $P=0.001$ ; PERMANOVA). The PERMANOVA analysis found that only the inoculation treatment had a significant effect on the community assembly within the roots of the wheat plants and explained 56% of the variation within the communities. PERMANOVA found no significant differences in community assembly between the wheat cultivars ( $F=1.3$ ,  $d.f.=2,42$ ,  $P=0.283$ ; PERMANOVA).

**Table 3.4:** Summary of two-way ANOVA (LM) results testing the effects of inoculation with *R. irregularis* on T-RF richness and diversity in three wheat cultivars. Bold indicates significant differences.

Variable	AMF treatment		Wheat cultivar		AMF Cultivar <sup>x</sup>	
	F	P	F	P	F	P
Degrees of freedom	d.f	1, 42	d.f	2, 42	d.f	2, 42
T-RF richness	1.3	0.259	1.3	0.289	4.4	<b>0.018</b>
Shannon diversity index	0.1	0.775	1.9	0.156	0.4	0.650



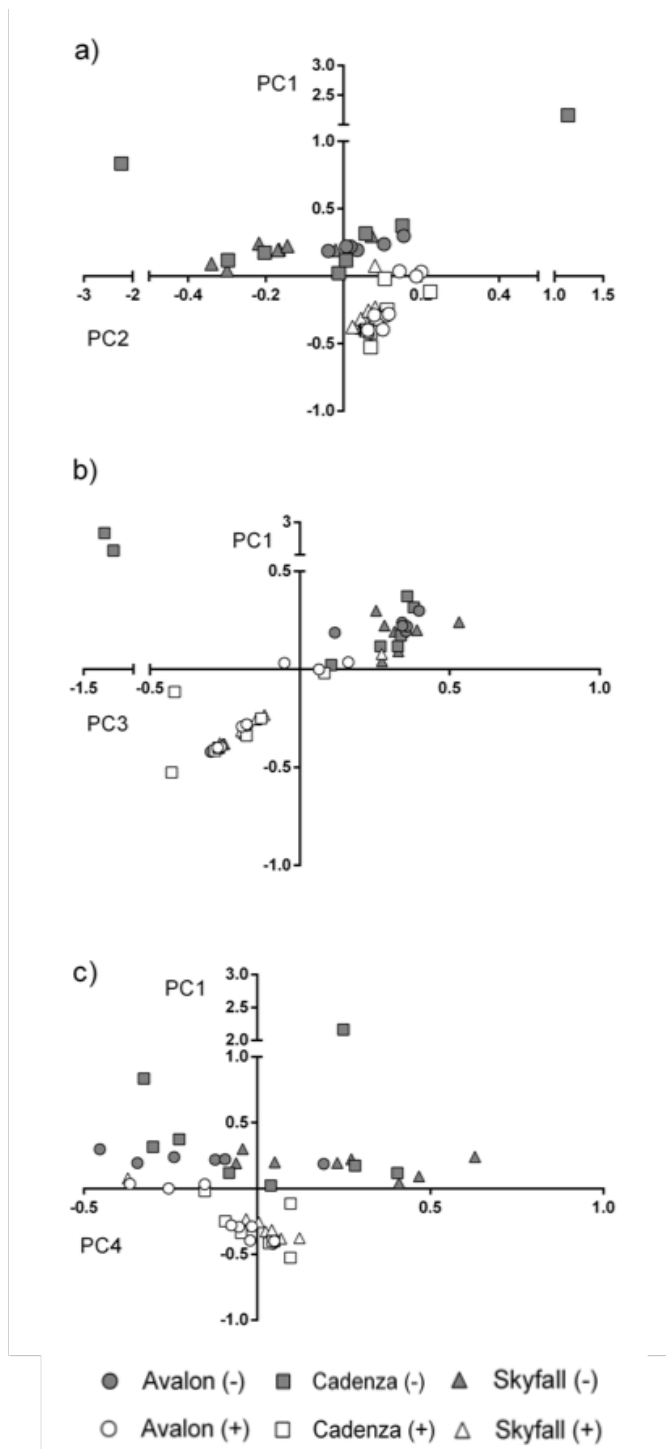
**Figure 3.4:** The effect of adding a *R. irregularis* commercial inoculum on the richness and diversity of T-RFs in the plant roots. (a) Shannon diversity index on the relative proportion of T-RFs in wheat root systems, (b) T-RF richness, based on the total number of T-RFs found in each wheat root system. Plants were all grown in non-sterile soil in the presence of a native AMF community and were then further inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (+) or a sterilised control (-). Experiments were conducted in three wheat cultivars, Skyfall (black bars), Avalon (grey bars) and Cadenza (white bars). Error bars represent the standard error of the mean. Different letters refer to significant differences between treatments. (n=8, P<0.05, two-way ANOVA, TukeyHSD post-test).

The ordination plots from the principal components analysis show separation of the different communities between the native AMF treatment and those in the native AMF + inoculant treatment (Figure 3.5). The first 4 principal components (PC) were found to explain 82% of the variation between treatments, and

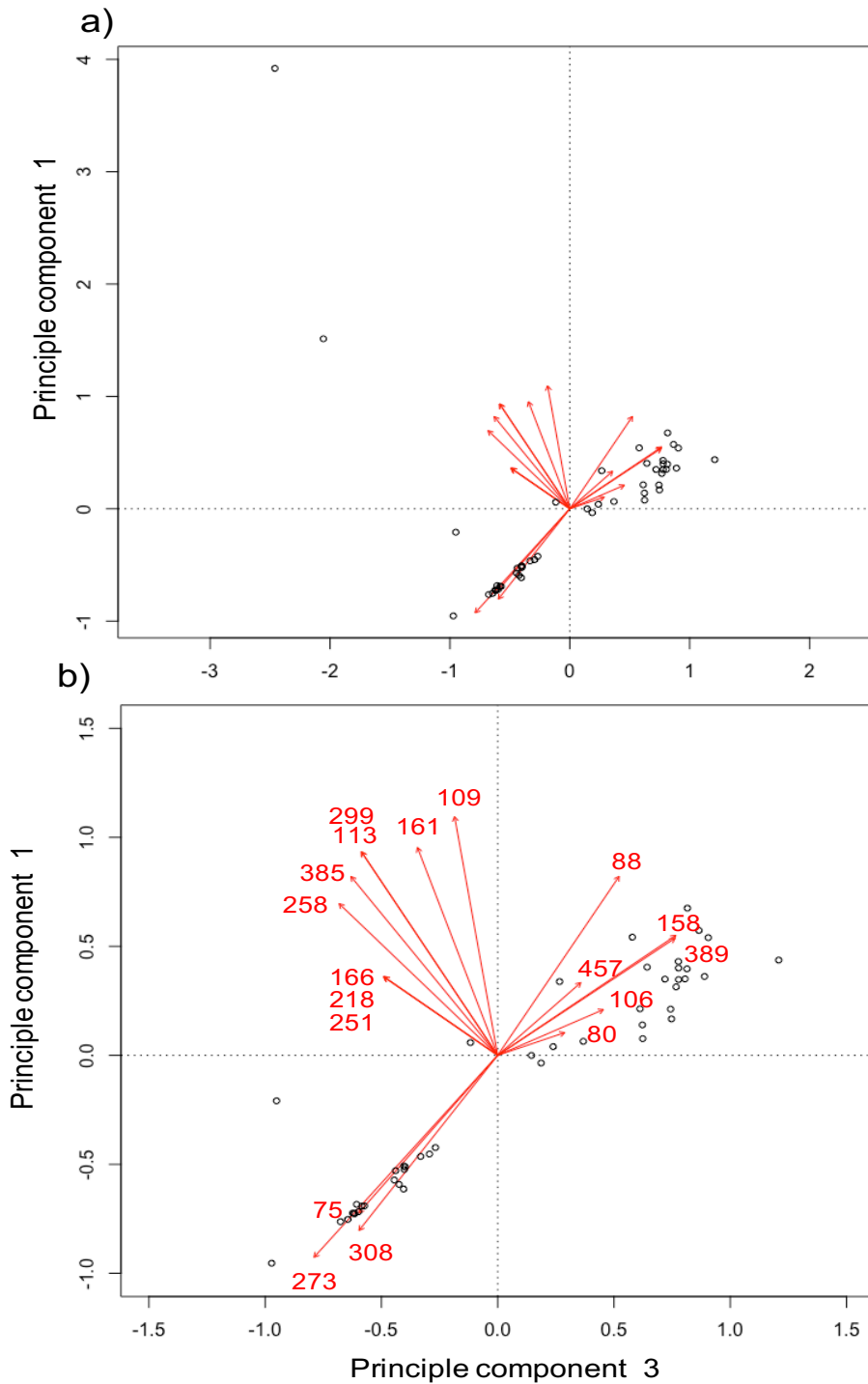
therefore these were the focus of my analysis. PC1 and PC3 which explained 30% and 19% of variation respectively, were significantly affected by the inoculation treatment (PC1:  $F=28.9$ ,  $d.f.=1,42$ ,  $P<0.001$ ; PC2:  $F=14.7$ ,  $d.f.=1,42$ ,  $P<0.001$ ; two-way ANOVA).

Figure 3.5 shows PC1 vs PC3 on a biplot, it is clear that many T-RFs drive the differences between communities. T-RFs '273', '308' and '75' are all more present in the inoculated plants. When samples of the inoculum were analysed, they contained two consistent peaks at '273' and '75' base pairs (bp), peak '75' represents 8% of sample fluorescence on average, whereas peak '273' represents 92% of sample fluorescence. The inoculum used in the experiment was advertised to contain a single species - *R. irregularis*. Therefore, sequences of *R. irregularis* were downloaded from GenBank and virtually digested with restriction enzymes; HpyCHIV produced a T-RF of  $273 \pm 1$  bp. This suggests *R. irregularis*, which was present only sporadically in non-inoculated plants (usually making up <5% of sample fluorescence), became much more frequent after *R. irregularis* inoculum was added. The T-RF at 308 bp was not present in the commercial inoculum, and only appeared in the un-inoculated plants infrequently. However, it appeared in every inoculated plant.

Many more T-RFs were indicators of the non-inoculated AMF community structure (Figure 3.6). A T-RF at 389 bp made up the highest proportion of fluorescence in the non-inoculated plants. Sequences of AMF species commonly associated with agricultural soils were downloaded and virtually digested, and both *F. mosseae* and *F. caledonium* produced T-RFs at 389 bp. Although T-RFs at 389 bp were also present in almost all inoculated samples, it made up a much lower proportion of sample fluorescence (average: 11%) compared to in the plants grown with the native AMF only (average: 52%). T-RF '88' also made up a high proportion of fluorescence in the non-inoculated plants (average: 22%) and had a reduced fluorescence in inoculated plants (average: 11%).



**Figure 3.5: Ordination plots based on principal components 1-4, representing T-RFLP data of plant root AMF communities.** Symbols represent individual replicates in the following treatments: grey symbols represent the plants grown in the presence of the native AMF community only, white symbols represent the AMF communities of plants grown in soil with the indigenous community and supplemented with a *R. irregularis* commercial inoculum. The shapes represent the three different wheat cultivars tested, Avalon (circle), Cadenza (square), Skyfall (triangle). The distance between the symbols represents the dissimilarity of their T-RF composition, measured by their Euclidean distance.



**Figure 3.6: Biplot based on the principal component analysis of the effects of inoculation with *R. irregularis* on the AMF community structure in the roots of three wheat cultivars.** The points on the biplot represent individual samples. The distance among samples approximates the dissimilarity of their T-RFs. The biplot displays T-RFs, and the distance of T-RFs from the origin indicates their relative importance in the biplot. (a) represents the original biplot with all samples represented, including two outlier samples, (b) is the same plot but zoomed in for clarity with the T-RFs labelled.

PC2 explained 24% of the variation among samples but was not found to be significantly affected by inoculation treatment or wheat cultivar (Table 3.5). The results of PC2 were highly driven by T-RFs which were only present in very few samples, including two outlier samples. PC4 was the only PC which was significantly affected by the wheat cultivar and explained only 8% of the variation between samples. The cultivar differences were driven by variations in AMF community composition within the roots of uninoculated cv. Avalon and Skyfall plants (TukeyHSD  $P < 0.05$ ).

**Table 3.5:** Summary of two-way ANOVA (LM) results from Principal Component scores testing the effects of inoculation with *R. irregularis* on the AMF community structure in the roots of three wheat cultivars.

<b>Variable</b>	<b>AMF treatment</b>		<b>Wheat cultivar</b>		<b>AMF x Cultivar</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	1, 42	<i>d.f</i>	2, 42	<i>d.f</i>	2, 42
PC1	28.9	<b>&lt;0.001</b>	1.1	0.330	2.7	0.078
PC2	2.7	0.105	0.6	0.532	0.3	0.753
PC3	14.7	<b>&lt;0.001</b>	2.5	0.091	0.8	0.421
PC4	1.9	0.177	5.7	<b>0.006</b>	2.4	0.105



### 3.5 Discussion

After inoculation with a commercially-available *R. irregularis* inoculum, AMF communities within the wheat roots were significantly altered in all cultivars. Despite the very different communities hosted in inoculated and non-inoculated plants, the overall species richness and diversity were similar between the two treatments. Inoculated wheat cultivars hosted very similar AMF communities, and had higher root length colonisation than non-inoculated wheat plants. However, the changes in AMF community composition and root colonisation did not result in enhanced P uptake within the plant tissue. This result differs substantially from Chapter 2 where P uptake was enhanced in all wheat cultivars after inoculation. The use of commercially-available inoculum also resulted in a slight growth depression in all wheat cultivars, which was not present in Chapter 2.

This study showed that the addition of a commercial inoculant to agricultural soil led to higher root colonisation in the wheat cultivars tested, although there were cultivar differences present in the extent of colonisation. This suggests that despite concerns based on previous research that commercial inoculants are often not viable (either through low-quality control or failure to establish in the new environments) (Vosátka et al., 2012; Berruti et al., 2013; Faye et al., 2013), the *R. irregularis* strain within this inoculum was compatible with the target environment and appeared able to compete with the native AMF community to colonise the host roots under the conditions used for the experiment. Wheat root colonisation by the species within the commercial inoculum (*R. irregularis*) is further supported by the T-RFLP analysis which showed an increase in the T-RF identified as *R. irregularis* after inoculation.

There was no differences in the Shannon diversity index between inoculated and non-inoculated plants or between the different wheat cultivars. Shannon diversity index measures community richness (i.e. absolute number of types in the community) and community evenness (i.e. how equal the abundances are between types). Richness remained unaffected by inoculation with *R. irregularis* in c.v. Skyfall and Avalon but was lower in inoculated c.v. Cadenza plants (6 T-RFs) compared to when plants were grown in the presence of the native AMF community only (8 T-RFs). However, it is important to note that T-RFLP is likely to underestimate species richness as multiple species can be represented within a T-RF. A reduction in community richness upon inoculation may be due to

inoculum species often being competitive colonisers, which may outcompete the species in the native community (Hart et al., 2017). Reductions in community diversity after use of AMF inoculants has been observed previously (Mummey et al., 2009; Koch et al., 2011), however, effects are not consistent with enhancements (Alguacil et al., 2011), and no changes in AMF diversity in the roots (Antunes et al., 2009) also reported.

Agricultural soils are often already low in diversity and have a few dominant species resulting in low community evenness. My results suggest adding a *R. irregularis* commercial inoculant may displace the dominant members of the native community but has no effect on overall diversity. The T-RF identified as *R. irregularis* represented a much higher proportion of sample fluorescence in inoculated plants. Whereas, the dominant T-RF in non-inoculated plants represented a lower proportion of fluorescence in inoculated plants. Suppression of the dominant native AMF species, may be problematic in some scenarios if the native species is more mutualistic to the plant host than the introduced species (Klironomos, 2003; Pellegrino et al., 2011; Séry et al., 2016). In this study, the change in community upon inoculation could have been responsible for the small growth depression seen in all cultivars upon inoculation with *R. irregularis*. However, the minimal effects on AMF diversity may alleviate some concerns about declines in AMF-acquired benefits, due to a reduction in niche complementarity between AMF species as overall species numbers tended to remain similar (Jansa et al., 2008; Antunes et al., 2009; Verbruggen et al., 2012). The change in AMF communities between the roots of inoculated and non-inoculated plants had no-effect on the P uptake within the plant tissues. However, the long-term effects of introducing foreign AMF isolates into new environments still requires further investigation.

Despite limited changes in overall richness or diversity in the AMF communities, the community composition within the plant root system of inoculated plants was significantly different to plants that were not inoculated, with the T-RF most likely to represent *R. irregularis* becoming more dominant within the community. Shifts in AMF community assembly upon inoculation with *R. irregularis* has previously been shown in *Pisum sativum* L. (Jin et al., 2013). In contrast, adding a commercial inoculant containing *R. irregularis* to a non-sterile agricultural field soil had no effect on the structure of the AMF community within the roots of a *Zea*

*mays* L. host plant (Antunes et al., 2009). Although all the above studies used *R. irregularis* species, evidence is accumulating that isolates within species of AMF can be highly diverse (Angelard et al., 2010; Mensah et al., 2015; Koch et al., 2017; Chen et al., 2018). The variation in outcomes of community composition upon inoculation with *R. irregularis* reveals the complex nature of AMF interactions, which may depend on the different host plants, native AMF communities within the soil or the commercial inoculant used.

The AMF community structure after the addition of a commercial inoculum is a result of the interactions between AMF species, including both competition and facilitation (Callaway & Walker, 1997). My study suggests that both AMF treatments had very uneven communities, as each community had a dominant T-RF and most other T-RF were rare and not well represented within the community. Uneven communities have been found previously to be a common structure within AMF communities, which are typically dominated by one species which makes up around 40% of the species abundance in the community (Dumbrell et al., 2010b). Species abundance distribution patterns in the communities suggest AMF species may separate into different niches which are based on the species response to the soil or host plant environment (Dumbrell et al., 2010b). The over-dominance of one species, which was not consistent between sites, suggested stochastic processes may also play a role in the structure of AMF communities. The theory put forward suggests recruitment to a new site can often be a chance event, the first fungus to colonise the roots is 'in the right place at the right time' and then gains additional C. More C resources allows faster proliferation through the soil and uncolonised roots, leading to a positive feedback mechanism promoting this one species and leading to over-dominance.

A T-RF likely corresponding to *F. mosseae* or *F. caledonium* dominated the plants grown in the presence of native AMF communities only. Whereas, a T-RF likely corresponding to *R. irregularis* displaced the dominant native AMF species in the plant roots when inoculated with a *R. irregularis*. Additionally, although the increase in colonisation varied between the wheat cultivars, this could not be associated to differences in the ability of *R. irregularis* to colonise the different wheat cultivars. As even cv. Avalon which only had a small non-significant

increase in colonisation was dominated by the T-RF within the inoculum likely corresponding to *R. irregularis*.

These results suggest *R. irregularis* can outcompete *F. mosseae* and *F. caledonium* when a large population is added through inoculation. Higher spore densities near the root may act as a stochastic event which means the inoculum species is highly likely to be the first coloniser and gains a competitive advantage over the native species (Mummey et al., 2009; Dumbrell et al., 2010b; Werner & Kiers, 2015). Werner and Kiers (2015) showed that the second mycorrhizal fungus to arrive at a plant root had a significantly reduced abundance. Therefore, the placement of inoculum in the soil, close to the plant roots and the higher spore density could have given the species within the inoculum a competitive advantage and consequently reduced the relative abundance of the dominant native AMF species (Hepper et al., 1988). This suggests timing of AMF inoculation in the field could be critical to successful establishment and colonisation of the plant. Inoculation at the time of sowing would likely be most successful at increasing colonisation, therefore seed coating could also be promising. The dominance of *R. irregularis* over other AMF species has been shown in previous studies (Engelmoer et al., 2014; Werner & Kiers, 2015). However, Jansa et al. (2008) found *F. mosseae* was always the superior competitor when compared to *R. irregularis* in an agricultural setting. Again, these results may, in part, be explained by differences between AMF isolates which have high genetic variability.

However, it is important to note that the T-RF (273), likely representing *R. irregularis*, was present in some non-inoculated plants and therefore is present in the agricultural soil, albeit at a lower spore density. *R. irregularis* may be less competitive in the non-inoculated plants due to a lower disturbance tolerance compared to species such as *F. mosseae* and *F. caledonium*, leading to reduced spore densities in the soil (Jansa et al., 2002; Oehl et al., 2003; Rosendahl, 2008). This highlights the importance of longer-term studies in the field to monitor inoculum establishment and survival over multiple years. This experiment was also carried out under semi-controlled greenhouse conditions, in field conditions where environmental conditions are more variable different AMF could have been favoured or fungal partners may have changed throughout the growing season (Bennett et al., 2013).

The changes in community composition in the inoculated plants in this study were driven by three T-RFs, reinforcing the importance of community analysis after inoculation and not presuming that the increase in fungal colonisation in the root system is exclusively the species within the inoculum. Instead, other fungal community changes could occur through facilitation interactions. *R. irregularis* has been shown in previous studies to facilitate other AMF species such as *Gigaspora* sp. (Thonar et al., 2014) and *Diversispora aurantia* (Symanczik et al., 2015). Facilitation may be due to the relatedness of species, as more closely related species have been shown to compete more strongly (Maherali & Klironomos, 2007, 2012; Mummey et al., 2009). More distantly related species may reduce competitive pressure by colonising distinct zones within the root or the soil. Within this study, a T-RF not present in the samples of inoculum tested had an enhanced presence in plants inoculated with *R. irregularis*, suggesting some facilitation interactions may be present. The mechanisms behind facilitation are unknown and require further investigation. However, it has been suggested that the first colonising fungus may create a more favourable environment for other species through suppressing the immune response of the plant or creating a more favourable host plant through enhancing plant nutritional status and potentially increasing C supply below ground (Thonar et al., 2014).

There was a small amount of evidence for non-random associations between the host and AMF, as community separation was found between cv. Avalon and Skyfall in the non-inoculated treatment. However, cultivar differences explained only a small proportion of variation in AMF communities (8%, PC4), ultimately suggesting that the limited species pool sampled within this arable system were mostly generalists, able to colonise wheat plants of many varieties. AMF are biotrophic, so there is likely a strong selective advantage of being non-specific in their associations and being able to gain C from many plant hosts (Smith & Read, 2008). AMF are also known to form common mycelium networks associating with multiple plant species simultaneously (Workman & Cruzan, 2016), which reinforces the general lack of host specificity within AMF species. However, plants co-occurring in the same natural grassland, or woodland ecosystems host different AMF communities within their roots, providing evidence host preferences may exist (Helgason et al., 2002; Vandenkoornhuysen et al., 2002). Arable systems characterised by crop rotations, often of annual plant species and heavy disturbance, are likely to select for generalists and non-specificity.

In this study, the *R. irregularis* inoculant did not enhance plant biomass; in fact, small decreases in shoot biomass were observed ( $F=6.23$ ,  $d.f.=1,42$ ,  $P=0.017$ ; two-way ANOVA). There were also no changes in plant P uptake with *R. irregularis* inoculum ( $F=0.57$ ,  $d.f.=1,42$ ,  $P=0.456$ ; two-way ANOVA). Increases in plant tissues [P] are likely related to the decreases in shoot biomass ( $F=9.73$ ,  $d.f.=1,42$ ,  $P=0.003$ ; two-way ANOVA). The three wheat cultivars tested generally had similar responses to inoculation within this study. These results contrast with the results in Chapter 2, where the plants had higher P uptake and [P] within the plant tissue after inoculation. The functionality of the symbiosis between AMF and plant hosts is known to be highly variable. However, the difference in plant responses between the studies is surprising due to highly similar growth conditions, including the wheat cultivars and commercial inoculum. The non-sterile agricultural soil was collected at different times of the year, and this could have had an effect on the native AMF community within the soil and the amount of AMF spores in the soil (Daniell et al., 2001; Cotton et al., 2015). The aim of applying commercial AMF inoculants is to enhance plant-derived benefits from AMF, such as improved nutrient uptake, which could increase crop yields or reduce chemical fertiliser applications. Therefore, if intra/interannual variation in AMF soil communities can substantially alter host plant responses to inoculation, this could have implications for their use by farmers, as major concerns could be raised about their reliability.

Additionally, although cultivar responses to inoculation in terms of growth and P uptake were generally similar within this experiment, when carbon-for-nutrients exchange within the symbiosis was measured in the previous chapter, there were substantial cultivar differences, with only cv. Avalon receiving less  $^{33}\text{P}$  and  $^{15}\text{N}$  from a fungal partner after inoculation with *R. irregularis*. From this study, we can confirm that it is most likely that cv. Avalon has a cultivar-specific response to *R. irregularis*, rather than a different AMF community colonising the plant roots, suggesting *R. irregularis* may not be a favourable symbiont for all wheat cultivars. Research should continue to pursue small-scale tests on inoculant suitability to a range of plant hosts and environments.

### **3.6 Conclusion.**

My results show that the AMF inoculant used effectively boosted wheat root colonisation without altering AMF community richness and diversity. These results corroborate the results in the previous chapter that *R. irregularis* inoculum can successfully compete with native AMF communities in agricultural soil to colonise plant roots, probably due to low, naturally occurring densities of native AMF spores and propagules in the soil. However, my first hypothesis that the addition of *R. irregularis* inoculum to the soil would cause changes to the mycorrhizal community colonising the plants, leading to a decrease in diversity and the domination of *R. irregularis* was only partially confirmed. Although the community composition was altered extensively leading to the domination of *R. irregularis* in inoculated samples; community composition in plants grown in the presence of the native community was already low in diversity due to the probable domination of a few disturbance tolerant species. Therefore, the overall diversity of the AMF communities colonising the plant roots was not altered, however, the dominant native fungus was displaced.

This experiment has reaffirmed the complexity of interactions between AMF species, indicating both competition and facilitation can occur, and how community composition and structure is determined deserves further investigation. We observed that the increase in root colonisation in inoculated plants was mostly but not exclusively *R. irregularis*, as we found an increased presence of some native AMF species not present in the inoculant. Root colonisation rose to different extents in the different cultivars after inoculation. However, this could not be related to differences in AMF community composition within the wheat cultivars. There was some evidence to support that wheat would have genotype-specific AMF communities colonising the root system, as AMF communities were shown to vary between wheat cultivars in the un-inoculated wheat plants. However, inoculation with *R. irregularis* homogenised the communities.

Inoculants are not expected to be beneficial in all cases, and combined evidence from this chapter and the previous chapter suggest that when cv. Avalon is inoculated with *R. irregularis* AMF acquired P and N may be reduced compared to the native AMF community. Implementation of commercial inoculants without adequate testing could harm the reputation of AMF inoculants within the

agricultural sector. Measures of inoculants successful establishment should also be assessed through long term studies over multiple seasons. If inoculants are not well adapted to the environment, they could be outcompeted after the initial competitive advance of a substantial boost in spore density has subsided.

Our data suggest that inoculant producers should aim to create synergistic consortia of AMF tailored to different crops and environments for applications to be successful at increasing plant-derived benefits from AMF. This will be particularly important in the future, as our environment is predicted to change dramatically due to human-induced global warming. Changes to the climate are modelled to reduce agricultural yields at a time where we must increase food production to feed our growing population. However, evidence is lacking on how changes to the climate will impact the symbiosis between AMF and our crop plants.



## **Chapter 4 : How will increasing [CO<sub>2</sub>] affect carbon-for-nutrient exchange between arbuscular mycorrhizal fungi and wheat?**

### **4.1 Introduction**

The burning of fossil fuels and changing land-use patterns have led to a rapid increase in atmospheric CO<sub>2</sub> concentrations ([CO<sub>2</sub>]) from 280 ppm in pre-industrial times to predicted levels between 500-900 ppm by the end of this century (IPCC, 2018). The corresponding rises in temperature will lead to greater instability in weather patterns such as periodic drought, flooding and heat-waves (Rosenzweig et al., 2001; IPCC, 2018). Agriculture is hugely vulnerable to temperature increases and shifting precipitation patterns (Shiferaw et al., 2014; Miyan, 2015). Therefore future climate simulations have predicted a decrease in global crop production (Rosenzweig & Parry, 1994).

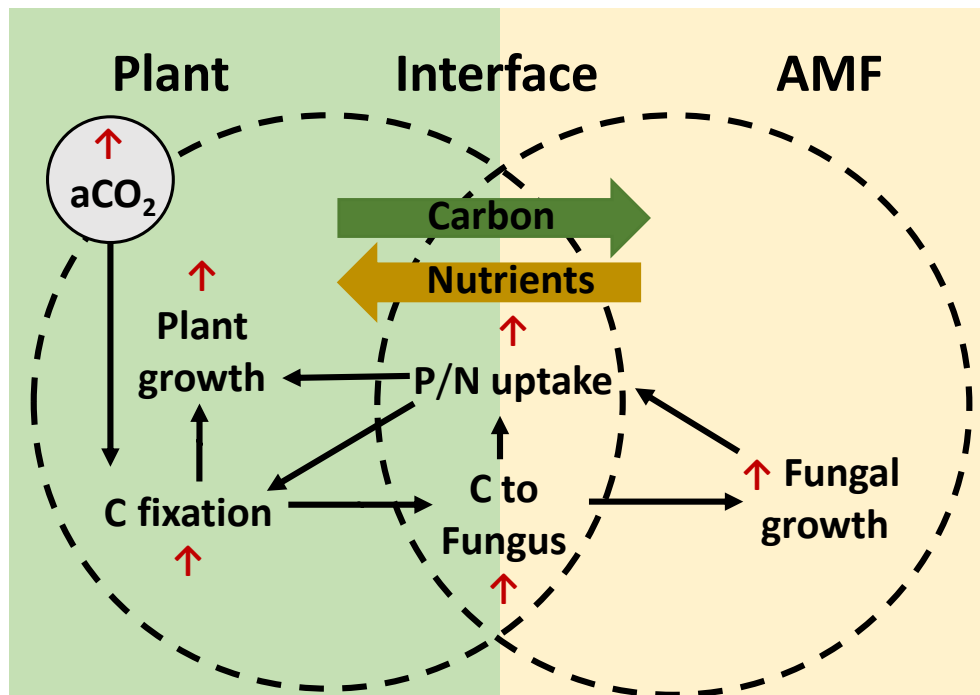
Elevated [CO<sub>2</sub>] could, in part, mitigate crop losses due to climate change by enhancing crop photosynthesis and water-use efficiency (Tubiello *et al.*, 1999; Manderscheid and Weigel, 2007; Rosenzweig and Parry 1994), specifically, in C3 plants such as wheat (see section: 1.1.2). The increase in plant growth or photosynthesis due to increasing [CO<sub>2</sub>] has been deemed the 'carbon fertilisation effect'. However, as plant growth increases, the need for essential nutrients such as N and P will also increase. Therefore, the extent of the 'carbon fertilisation' effect on crop productivity will depend on the level of plant-available nutrients in the soil (Cavagnaro, Gleadow, & Miller, 2011; Pandey et al., 2015a). Alternatively, inadequate C sink (i.e. from plant growth) may limit the plant's ability to use photosynthates, and a build-up of leaf starch can lead to a down-regulation of photosynthesis (Drake et al., 1997; Goicoechea et al., 2014; Gavito et al., 2019).

It is essential to take advantage of any opportunities to increase yields associated with climate change. However, if this involves higher inputs of fertilisers to gain the maximum [CO<sub>2</sub>] associated growth increases, we may exacerbate the problem of excessive fertiliser inputs in agriculture (see section 1.1.3). For example, increased fertiliser use would accelerate the depletion of rock phosphate reserves and exacerbate pollution of natural ecosystems, through run-off of fertilisers into water bodies (Cordell et al., 2009). It is therefore important to

investigate whether the 'carbon fertilisation' effect can be achieved under more nutrient-limited conditions (Pandey et al., 2015a).

As such, it is vital to consider the potential for a more beneficial relationship between AMF and C3 crops, under elevated [CO<sub>2</sub>]. Plant nutrition plays a major role in plant growth responses to elevated [CO<sub>2</sub>] (Pandey et al., 2015b). Therefore, growth rates are likely to be regulated by the symbiotic relationship between plants and AMF (Figure 4.1). If AMF biomass increases under elevated [CO<sub>2</sub>], there may also be an increase in belowground sink strength, decreasing the build-up of photosynthates and preventing down-regulation of photosynthesis (Fitter et al., 2000; Gavito et al., 2000, 2019; Baslam et al., 2012). Thus, it is critical to understand how the development and function of the symbiosis will change under elevated [CO<sub>2</sub>], and if this is wheat cultivar dependent. For example, in Chapter 2 the cultivars differed in the quantities of nutrients acquired via AMF. Cultivars which are able to acquire more nutrients through an AMF partner, i.e. cv Skyfall, may be able to capitalise the 'carbon fertilisation' effect. Alternatively, the cultivars also differed in the amounts of C allocated below-ground to an AMF partner. Where AMF act as a larger sink for C within a wheat cultivar, i.e. cv. Avalon, they may decrease the build-up of photosynthates and prevent down-regulation of photosynthesis at elevated [CO<sub>2</sub>].

The plant response to the symbiosis may change over time as the symbiosis develops and perhaps increases in C sink strength or nutrient acquisition which would enhance growth at elevated [CO<sub>2</sub>]. On the other hand, FACE experiments have shown that acclimation of the photosynthetic machinery can occur and lead to the downregulation of photosynthesis after extended periods of exposure to elevated [CO<sub>2</sub>] (Ainsworth & Long, 2005). Plant responses to AMF also have the potential to change over-time as plant nutrient requirements change; plants have highest nutrient demands when entering the reproductive growth phase (Abbate et al., 1995), and therefore may increase reliance on AMF partners for nutrient uptake.



**Figure 4.1: How elevated atmospheric CO<sub>2</sub> ([CO<sub>2</sub>]) and the symbiotic relationship with arbuscular mycorrhizal fungi (AMF) could interact to enhance plant growth responses to elevated [CO<sub>2</sub>].** In brief, elevated [CO<sub>2</sub>] enhances carbon (C) fixation and plant growth, but growth is limited by plant nutrient uptake. Enhanced C fixation may increase C allocated from the plant to the fungus, in turn increasing fungal growth and nutrient acquisition. Higher fungal acquired nutrients will relieve the limitations on C fixation and increase plant growth (Adapted from Pandey, Zinta, et al., 2015).

The balance of resources is likely to shift considerably as [CO<sub>2</sub>] rises in the future, C3 plants are predicted to fix more C and require greater acquisition of N and P. If the formation of AMs is C limited, elevated [CO<sub>2</sub>] may lead to improved AMF development within the roots and soil, which could, in turn, increase mycorrhizal nutrient acquisition. There has been a large number of studies on the effect of elevated [CO<sub>2</sub>] on AMF colonisation, with the results summarised in reviews and meta-analyses (Staddon & Fitter, 1998; Fitter et al., 2000; Treseder, 2004; Alberton et al., 2005). According to previous research, AMF colonisation has been found to increase, remain unaffected or, in a small number of cases decrease, in response to elevated [CO<sub>2</sub>]. However, the overall conclusions of a large meta-analysis suggest that both plants and AMF respond positively to elevated [CO<sub>2</sub>] by 25% and 21% respectively, the response parameter measured varied dependent on the study.

Plants have been shown to invest higher amounts of C below ground as [CO<sub>2</sub>] increases (Drigo et al., 2010, 2013; Field et al., 2012, 2015a), suggesting a possible rise in C supply to AMF symbionts which has been shown to increase mycorrhizal biomass (Drigo et al., 2007). It is essential to elucidate if this could increase nutrient acquisition by the plants in return.

Despite the noted increases in fungal biomass with elevated [CO<sub>2</sub>], whether this benefits the host plant has not been shown unambiguously. Greater fungal biomass could result in higher nutrient gain and transfer to the host as the hyphal network can forage more soil volume for nutrients. Conversely, increased fungal biomass may cause competition between the host and the fungus for vital nutrients and thereby drive a reduction in nutrient transfer from fungus to plant (Alberton et al., 2005). Whether the symbiosis provides enhanced nutrient acquisition to the plants could also change over time or be dependent on other environmental factors. Elevated [CO<sub>2</sub>] did not increase the contribution of the AMF pathway to P uptake in *M. truncatula*, *B. distachyon*, *Pisum sativum* (Gavito et al., 2002; Jakobsen et al., 2016). Previous studies in wheat have suggested neither acquisition of N or P by AMF increased at elevated [CO<sub>2</sub>] (Zhu et al., 2016; Thirkell et al., 2019). Therefore, this may indicate AMF expansion and function were not regulated or limited by C resources at ambient [CO<sub>2</sub>] (Jakobsen et al., 2016).

Alternatively, the impact of [CO<sub>2</sub>] enrichment may vary widely between plant species and AMF species. Indeed, a study across 14 plant species and five AMF communities, found rising [CO<sub>2</sub>] could either increase or decrease plant benefits from AMF (Johnson et al. 2005). Similarly, AMF's effect on 'carbon fertilisation' has been variable, with growth responses to elevated [CO<sub>2</sub>] unaffected by AMF in some cases (Gavito et al., 2000, 2002, 2003; Jakobsen et al., 2016), but amplified by AMF in other cases (Hartwig et al., 2002; Zhu et al., 2016).

Research which assesses carbon-for-nutrient exchange within the symbiosis has shown that the nutrient exchange efficiency is affected by plant species and [CO<sub>2</sub>] concentrations (Field et al., 2012). In non-vascular liverworts, AMF symbiotic efficiency (i.e. the amount of P gained by the plant per unit of C allocated to the fungal symbiont) improves at higher [CO<sub>2</sub>] concentrations (1,500ppm). At elevated [CO<sub>2</sub>] increased P gain by the plant shoots far outweighs any increase in C allocation to the fungal partner. However, the efficiency of the AMF symbiosis

in vascular plants such as ferns and angiosperms vastly decreases at elevated [CO<sub>2</sub>] (1500 ppm). This is due to the >1000% increase in C allocation to the fungal partner at elevated [CO<sub>2</sub>] but no increase in fungal acquired P to the plants (Field et al., 2012). This suggests increasing [CO<sub>2</sub>] may result in increased C allocation below ground to the mycorrhizal fungi but not necessarily be reciprocated through increased nutrient allocation to the plant by the fungal partner.

The effect of changing [CO<sub>2</sub>] concentrations on crop-mycorrhizal symbioses, particularly where this involves cereals, remains mostly unknown, with very few studies focusing on differences between crop cultivars. In the context of future climate change, this represents a significant knowledge gap and a potential barrier to future sustainability in agriculture; especially due to the increasing interest into the application of commercially available AMF inoculants in agricultural soils. AMF functioning can be highly context-dependent and be driven, among other things, by fungal ID and environmental factors (Munkvold et al., 2004; Hoeksema et al., 2010), we currently don't know how fungal inoculants, which could function well in current systems, will respond to future climates.

## 4.2 Aims and Hypotheses

The objective of this study was to quantify the ability of a commercially available inoculant containing mycorrhizal fungi *R. irregularis* to contribute to improving wheat growth responses to elevated [CO<sub>2</sub>] through enhanced nutrient acquisition and reduced cost of the symbiosis.

I investigated the efficiency of this inoculum at improving wheat nutrient uptake and growth at current day ambient [CO<sub>2</sub>] and an elevated [CO<sub>2</sub>] representative of a future atmosphere in 2070 should steps not be taken in curbing global CO<sub>2</sub> emissions (IPCC, 2019). I used <sup>14</sup>C, <sup>33</sup>P and <sup>15</sup>N isotopes, to assess how elevated [CO<sub>2</sub>] alters carbon-for-nutrients exchange within the symbiosis, due to predicted heightened plant nutrient requirements at elevated [CO<sub>2</sub>], alongside enhanced plant C resources.

I test the following hypotheses:

1. Wheat, due to being a C3 crop, will have improved C fixation at elevated [CO<sub>2</sub>], and this will drive greater C allocation to fungal symbionts.
2. Increased C allocation below ground will result in more abundant fungal biomass in terms of percentage root colonisation and extra-radical mycelium in the soil.
3. Increased AMF biomass will result in increased plant nutrient acquisition through the fungal partner.
4. The 'carbon fertilisation' achieved under elevated [CO<sub>2</sub>] will be further enhanced by AMF associations in a low nutrients environment.
5. Plant responses to AMF and atmospheric [CO<sub>2</sub>] will change over time, for example, plants will acclimate to elevated [CO<sub>2</sub>], and as plants enter the reproductive growth stage, they will have an increased nutrient demand, leading to more benefits from having an AMF partner.
6. Wheat will have cultivar-specific responses to inoculum additions and rising atmospheric [CO<sub>2</sub>]. Cultivars which acquire more nutrients through AMF partners, for example, cv. Skyfall in the previous experiment (Chapter 2) will have higher biomass increases at elevated atmospheric [CO<sub>2</sub>].

## **4.3 Materials and Methods**

### **4.3.1 Biological material and growth conditions**

Wheat seeds (*Triticum aestivum* L.) were sterilised with chlorine gas and subsequently germinated in Petri dishes on damp filter paper at 20°C for six days. Seedlings were then transplanted into a sand and perlite substrate mix (3:1) in plant pots (1100 cm<sup>3</sup>).

Wheat plants were grown in a controlled environment growth cabinet (Snijders labs, MicroClima-series 1200, Netherlands) at 20°C day temperature and 15°C night temperature, under a 16-h/8-h light/dark photoperiod, relative humidity of 70%, at either ambient (440 ppm) or elevated (800 ppm) [CO<sub>2</sub>]. The seedlings were inoculated with either 20 g of a commercially available AMF inoculum containing a single species *R. irregularis* (PlantWorks Limited, Kent) or an autoclaved control. The inoculum is produced in vivo using host plants clover and maize. The growing substrate is a mixture of pumice and zeolite (1:1 blend). The inoculum contains a mix of infective propagules including colonised root fragments, hyphae and AMF spores. The inoculum was placed in the potting hole at the time of planting (see further detail below). Plants were watered every three days and given 30 ml of 40% nitrate type Long Ashton solution weekly (Hewitt, 1966).

#### **4.3.1.1 Experiment 1: Assessing the interaction between AMF and [CO<sub>2</sub>] on wheat physiology**

When assessing the interaction between AMF and [CO<sub>2</sub>] on wheat physiology, the plants (cv. Skyfall, Avalon and Cadenza) were grown in Jumbo root trainer pots (Haxnicks Tildenet) (24cm x 6.5 cm x 7 cm) and harvested at eight week and ten week time points (n=5 per experimental treatment). These time-points were chosen as preliminary tests had shown extensive colonisation had formed by the eight week time-point and subsequent time-points of ten weeks allowed assessment of how the effect of AMF and [CO<sub>2</sub>] on wheat changed over time as the plants entered the reproductive growth stage, where plant nutrient demand substantially increases.

The effect of [CO<sub>2</sub>] and AMF inoculation was measured on plant biomass, plant tissue [P] and percentage root length colonised with AMF at both the 8 and 10 week timepoint, using the methods described previously. At harvest, the

substrate was carefully washed from the roots with water. Fresh weight of roots was recorded for each plant harvested, and a subsample of roots was stored in 50% ethanol at 4°C until colonisation analysis. The shoot material and remaining root tissue were freeze-dried, and dry weights recorded. The total root dry weight could be quantified using the proportion of fresh weight to dry weight of the dried subsample.

### **4.3.2 Experiment 2: Measuring the efficiency of wheat-AMF symbiosis through isotope tracing experiments (<sup>33</sup>P, <sup>15</sup>N & <sup>14</sup>C)**

#### **4.3.2.1 Experimental set-up**

Plant pots with the same soil volume (1100 cm<sup>3</sup>) were used during experiments quantifying <sup>33</sup>P and <sup>15</sup>N for <sup>14</sup>C exchange (n=12 per experimental treatment) (cv. Skyfall and Avalon). Cv. Skyfall and Avalon were chosen for the isotope tracing experiments as in experiment 1 they were more responsive to changes in [CO<sub>2</sub>] and AMF than cv. Cadenza.

The wheat plants were grown for eight weeks, in plant pots with an 1100 cm<sup>3</sup> soil volume, as described above (n=12 per experimental treatment). The carbon-for-nutrients exchange within this experiment was measured by the same method as described in Chapter 2, via labelling patches of soil within fungal access only plastic cores, with isotopes <sup>33</sup>P-Phosphoric acid (1MBq; Hartmann Analytic, Germany) (Specific activity: cv. Skyfall – 118.8 TBq mmol<sup>-1</sup>, Avalon – 183.2 TBq mmol<sup>-1</sup>) and <sup>15</sup>N ammonium chloride (1.5mg/ml) (MP Biomedicals, Santa Ana, USA) (see section 2.3.2.2).

Diffusion of <sup>33</sup>P and <sup>15</sup>N out of the cores and other microbial nutrient cycling was controlled for by rotating half of the labelled cores within a treatment as described in Chapter 2 (Fig.2). At the 8-week time point, plants were placed in sealed airtight chambers using large ziplock bags. Shoots were supplied with <sup>14</sup>CO<sub>2</sub> within a sealed system for a 16-h photoperiod via the liberation of 110 µl Sodium bicarbonate [<sup>14</sup>C] 1 MBq (specific activity: all cultivars - 2.13 GBq mmol<sup>-1</sup>; Hartmann Analytic, Germany) (see section 2.3.2.2 for details).

#### **4.3.2.2 Plant harvest and tissue analysis**

Upon completion of the 16-h labelling period, plants were harvested by separation of all plant and soil material into the following parts: static core soil,



rotated core soil, bulk soil, shoot biomass and root biomass. A sample of the bulk soil was stored at 4°C for assessment of hyphal density, and a subsample of the root system was stored in 50% ethanol for assessment of AMF root colonisation. All components were freeze-dried and weighed. The effect of [CO<sub>2</sub>] on the efficiency of wheat-AMF symbiosis was measured through analysis of the harvested components for the content of <sup>33</sup>P, <sup>15</sup>N and <sup>14</sup>C, using methods described previously (See section 2.3). Plant biomass, plant tissue [P], percentage root length colonised with AMF, and AMF hyphal density in the soil were also measured using methods described previously (See section 2.3).

### **4.3.3 Statistical analysis.**

For experiment 1, the impact of AMF treatment and [CO<sub>2</sub>] on measured parameters, and whether effects differed between three wheat cultivars was assessed by three-way ANOVA followed by TukeyHSD tests using Minitab (Version 17). Differences in percentage colonisation between ambient and elevated [CO<sub>2</sub>] within three wheat cultivars were measured by performing a two-way ANOVA followed by TukeyHSD tests using Minitab (Version 17). Before statistical analysis, data were checked for confirmation to assumptions of normality and equal variance using normal probability plots and residuals vs fits plots.

Where data did not meet the assumptions as was the case for root biomass (eight weeks), shoot [P] (eight weeks), percentage vesicles (ten weeks), shoot biomass (ten weeks), root biomass (ten weeks), shoot absolute P (ten weeks) the data was transformed using the optimal lambda function in Minitab (Version 17), to fulfil data assumptions.

Within experiment 2 which measured carbon-for-nutrient exchange through isotope tracing, the impact of [CO<sub>2</sub>] on measured parameters, and whether effects differed between wheat cultivars Avalon and Skyfall was assessed by two-way ANOVA followed by TukeyHSD tests using Minitab (Version 17). Before statistical analysis, data were checked for confirmation to assumptions of normality and equal variance using normal probability plots and residuals vs fits plots.

In the cases of shoot biomass, root biomass, percentage root length colonisation, extra-radical hyphal lengths, root [P], shoot [C], root [C], and absolute shoot <sup>15</sup>N

the data were transformed using the optimal lambda function in Minitab (Version 17), to fulfil data assumptions.

The homoscedasticity of the concentration and total  $^{33}\text{P}$  data in the shoots and the roots could not be fixed by transforming the data. The normality and homoscedasticity of C allocated to fungi in the cores and C assigned to fungi in the pots could not be fixed by transformation. Therefore, Kruskal-Wallis tests were performed across the four treatments (e.g. ambient or elevated  $[\text{CO}_2]$ ) within each wheat cultivar Avalon and Skyfall) on Minitab (Version 17). A post-test Dunn's multiple comparison test was performed using a Minitab Macro (KrusMC.mac) in Minitab (Version 18).

## 4.4 Results

### 4.4.1 Experiment 1: The interaction between [CO<sub>2</sub>] and AMF symbiosis.

#### 4.4.1.1 Root colonisation.

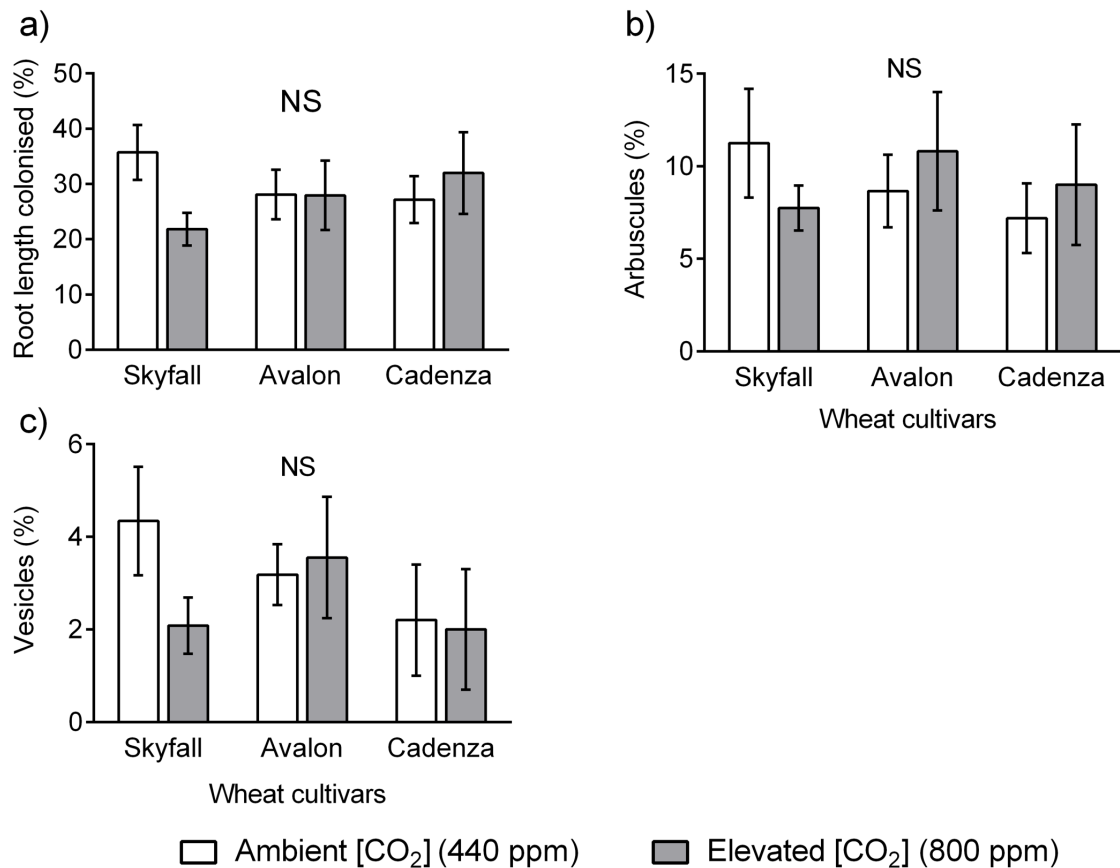
AMF colonisation was well established within all cultivars and ranged between 8-57% at the eight week time point and between 14-66% at the ten week time point. Plants in the non-mycorrhizal treatment remained uncolonized. The rise in [CO<sub>2</sub>] from present-day ambient levels (440 ppm) to predicted future levels (800 ppm) had no significant effect on mycorrhizal colonisation within the roots in any of the wheat cultivars. This held true for both the eight week timepoint ( $F=0.52$ ,  $d.f.=1,24$ ,  $P=0.479$ ; two-way ANOVA, Table 4.1, Figure 4.2a) and when the experiment was repeated to a ten week timepoint ( $F=1.26$ ,  $d.f.=1,24$ ,  $P=0.218$ ; two-way ANOVA, Table 4.1, Data not shown).

**Table 4.1:** Summary of two-way ANOVA (GLM) results testing the effects of [CO<sub>2</sub>] concentrations on the root colonisation of three wheat cultivars inoculated with *R. irregularis*. Significant values highlighted in bold.

	<b>Variable</b>	<b>[CO<sub>2</sub>] treatment</b>		<b>Wheat cultivar</b>		<b>[CO<sub>2</sub>] x Cultivar</b>	
		<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Time point</i>	<i>Degrees of freedom</i>	<i>d.f.</i>	<i>1, 24</i>	<i>d.f.</i>	<i>2,24</i>	<i>d.f.</i>	<i>2,24</i>
8 weeks	Root colonisation (%)	0.52	0.479	0.04	0.956	1.7	0.203
	Arbuscules (%)	0.01	0.942	0.24	0.785	0.78	0.469
	Vesicles (%)	0.62	0.437	0.82	0.454	0.82	0.454
<i>Time point</i>	<i>Degrees of freedom</i>	<i>d.f.</i>	<i>1, 23</i>	<i>d.f.</i>	<i>2,23</i>	<i>d.f.</i>	<i>2,23</i>
10 weeks	Root colonisation (%)	1.26	0.218	1.63	0.218	0.95	0.400
	Arbuscules (%)	1.33	0.261	3.49	0.048	1.19	0.322
	Vesicles (%)	0.32	0.579	1.34	0.283	1.08	0.356

Similarly, at the eight week time point there was no change in the presence of fungal structures, arbuscules and vesicles, within plant root systems between ambient and elevated [CO<sub>2</sub>] (% arbuscules at eight weeks:  $F=0.01$ ,  $d.f.=1,24$ ,  $P=0.942$ , Table 4.1, Figure 4.2b) (% vesicles at eight weeks:  $F=0.62$ ,  $d.f.=1,24$ ,  $P=0.437$ , Table 4.1, Figure 4.2c). This was also the case at the ten weeks timepoint (ten weeks arbuscules:  $F=1.33$ ,  $d.f.=1,23$ ,  $P=0.218$ , Table 4.1, Data not

shown) (ten weeks vesicles:  $F=0.32$ ,  $d.f.=1,23$ ,  $P=0.579$ , Table 4.1, Data not shown).



**Figure 4.2: Percentage of the root length colonised by arbuscular mycorrhizal fungi at ambient or elevated atmospheric CO<sub>2</sub> concentrations ([CO<sub>2</sub>]).** (a) total colonisation, (b) arbuscules, (c) vesicles. All plants were inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* and grown at ambient [CO<sub>2</sub>] (440 ppm -white bars) or elevated [CO<sub>2</sub>] (800 ppm – grey bars). Error bars represent the standard error of the mean. NS – no significant difference ( $n=5$ ,  $P<0.05$ , two-way ANOVA, Tukey HSD post-test) between treatments.

#### 4.4.1.2 Plant biomass.

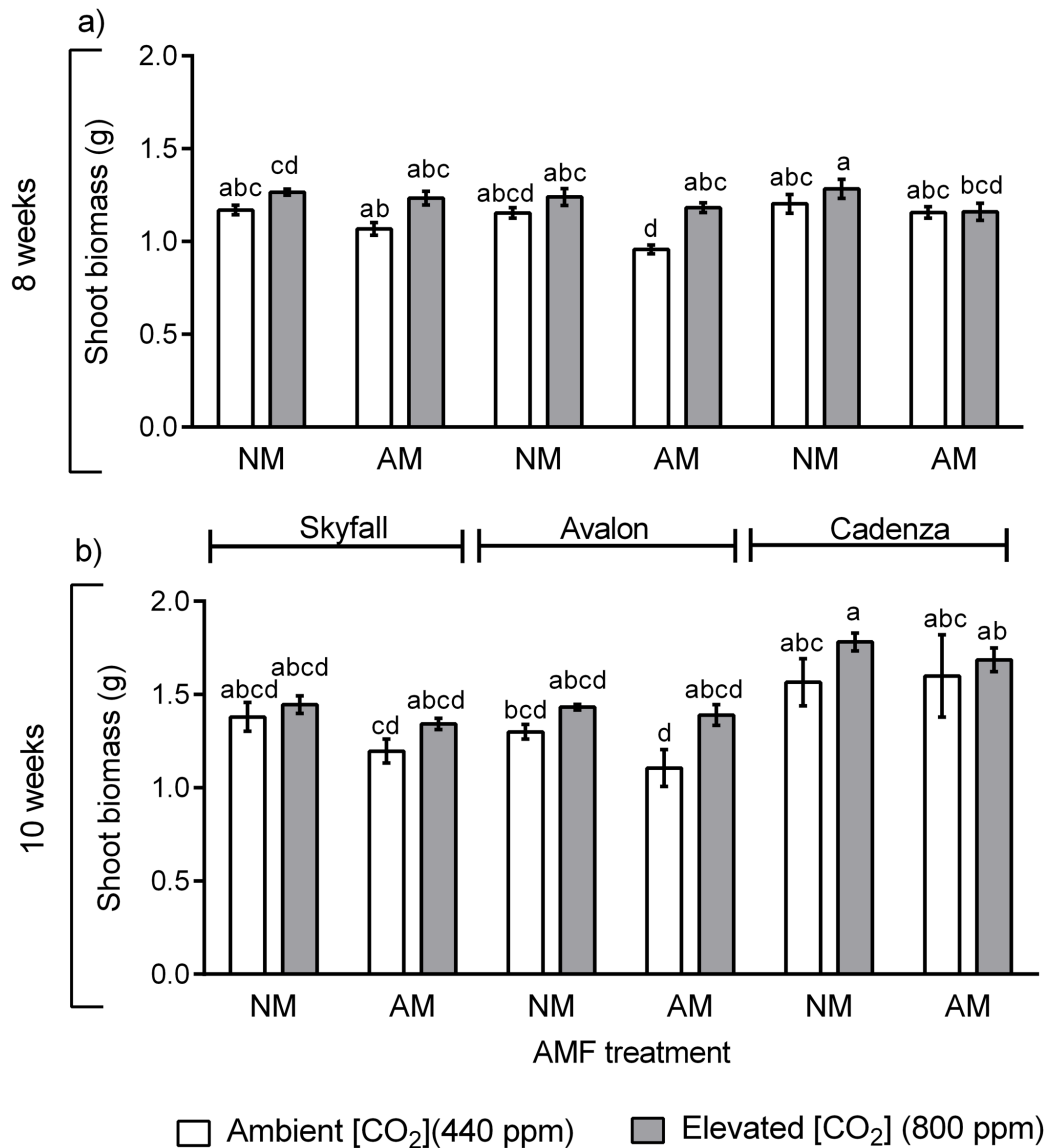
Shoot biomass was higher after eight weeks of growth in the elevated [CO<sub>2</sub>] treatment. Within cv. Skyfall and Avalon mycorrhizal plants had amplified biomass gains under elevated [CO<sub>2</sub>] compared to non-mycorrhizal plants, but this trend was not present in cv. Cadenza, suggesting how AMF effects plants response to [CO<sub>2</sub>] is cultivar dependent ([CO<sub>2</sub>] x AMF x Cultivar interaction:  $F=3.62$ ,  $d.f.=2,24$ ,  $P=0.034$ ; three-way ANOVA, Table 4.2, Figure 4.3a ). In cv. Skyfall elevated [CO<sub>2</sub>] concentrations had a positive effect on biomass in both

non-mycorrhizal and mycorrhizal treatments, with higher shoot biomass by 8% and 16% respectively. Similarly, within cv. Avalon elevated [CO<sub>2</sub>] concentrations had a positive effect on shoot biomass, in the non-mycorrhizal treatment biomass was slightly higher (7%), but the difference was much larger in the mycorrhizal treatment (23%). In contrast, although cv. Cadenza had slightly higher biomass under elevated [CO<sub>2</sub>] in the non-mycorrhizal treatment (7%), in the mycorrhizal treatment shoot biomass was no larger (-3%).

AMF also had a significant effect on wheat biomass. In cv. Skyfall AMF had a small but negative effect on shoot biomass. At ambient [CO<sub>2</sub>], biomass was 9% lower with AMF colonisation compared to non-mycorrhizal plants, whereas at elevated [CO<sub>2</sub>] it was only 2% less. Correspondingly in cv. Avalon, mycorrhizal plants had lower shoot biomass by 17% and 5% in ambient [CO<sub>2</sub>] and elevated [CO<sub>2</sub>] treatments respectively. However, cv. Cadenza showed the opposite pattern with mycorrhizal plants having 4% lower biomass in ambient [CO<sub>2</sub>] and 13% less in elevated [CO<sub>2</sub>] when compared to non-mycorrhizal plants.

**Table 4.2:** Summary of three-way ANOVA (GLM) results testing the effects of [CO<sub>2</sub>] and inoculation with *R. irregularis* on the biomass and tissue phosphorus concentrations of three wheat cultivar. Significant values highlighted in bold, where interactions were significant main effects were not reported.

	Variable	Wheat cultivar		[CO <sub>2</sub> ] treatment		AMF treatment		Cultivar x [CO <sub>2</sub> ]		Cultivar x AMF		[CO <sub>2</sub> ] x AMF		[CO <sub>2</sub> ] x AMF x Cultivar	
		F	P	F	P	F	P	F	P	F	P	F	P	F	P
weeks of growth		d.f.	2, 48	d.f.	1,48	d.f.	1,48	d.f.	2,48	d.f.	2,48	d.f.	2,24	d.f.	2,24
8 weeks	Shoot [P] (mg g <sup>-1</sup> )	77.27	<0.001	2.16	0.148	1.08	0.305	2.12	0.131	4.74	0.013	7.89	0.007	3.30	<b>0.045</b>
	Shoot P (mg)	82.95	<b>&lt;0.001</b>	12.11	<b>0.001</b>	32.73	<b>&lt;0.001</b>	3.14	0.052	1.16	0.321	2.85	0.098	0.69	0.504
	Shoot biomass (g)	1.94	0.155	16.55	<0.001	20.71	<0.001	4.14	0.022	0.72	0.490	0.13	0.725	3.62	<b>0.034</b>
	Root biomass (g)	3.01	0.059	3.71	0.060	3.40	0.071	2.33	0.108	0.38	0.684	1.18	0.282	1.40	0.257
10 weeks	Shoot [P] (mg g <sup>-1</sup> )	6.86	<b>0.002</b>	0.03	0.859	1.26	0.267	0.80	0.454	0.30	0.743	0.03	0.854	0.01	0.989
	Shoot P (mg)	39.95	<b>&lt;0.001</b>	14.57	<b>&lt;0.001</b>	11.91	<b>0.001</b>	2.06	0.139	0.59	0.560	1.40	0.242	0.97	0.386
	Shoot biomass (g)	17.79	<b>&lt;0.001</b>	12.31	<b>0.001</b>	5.18	<b>0.027</b>	0.51	0.602	0.60	0.555	0.52	0.473	0.74	0.481
	Root biomass (g)	1.56	0.221	0.00	0.990	1.77	0.190	0.62	0.542	1.74	0.187	0.49	0.489	0.06	0.938

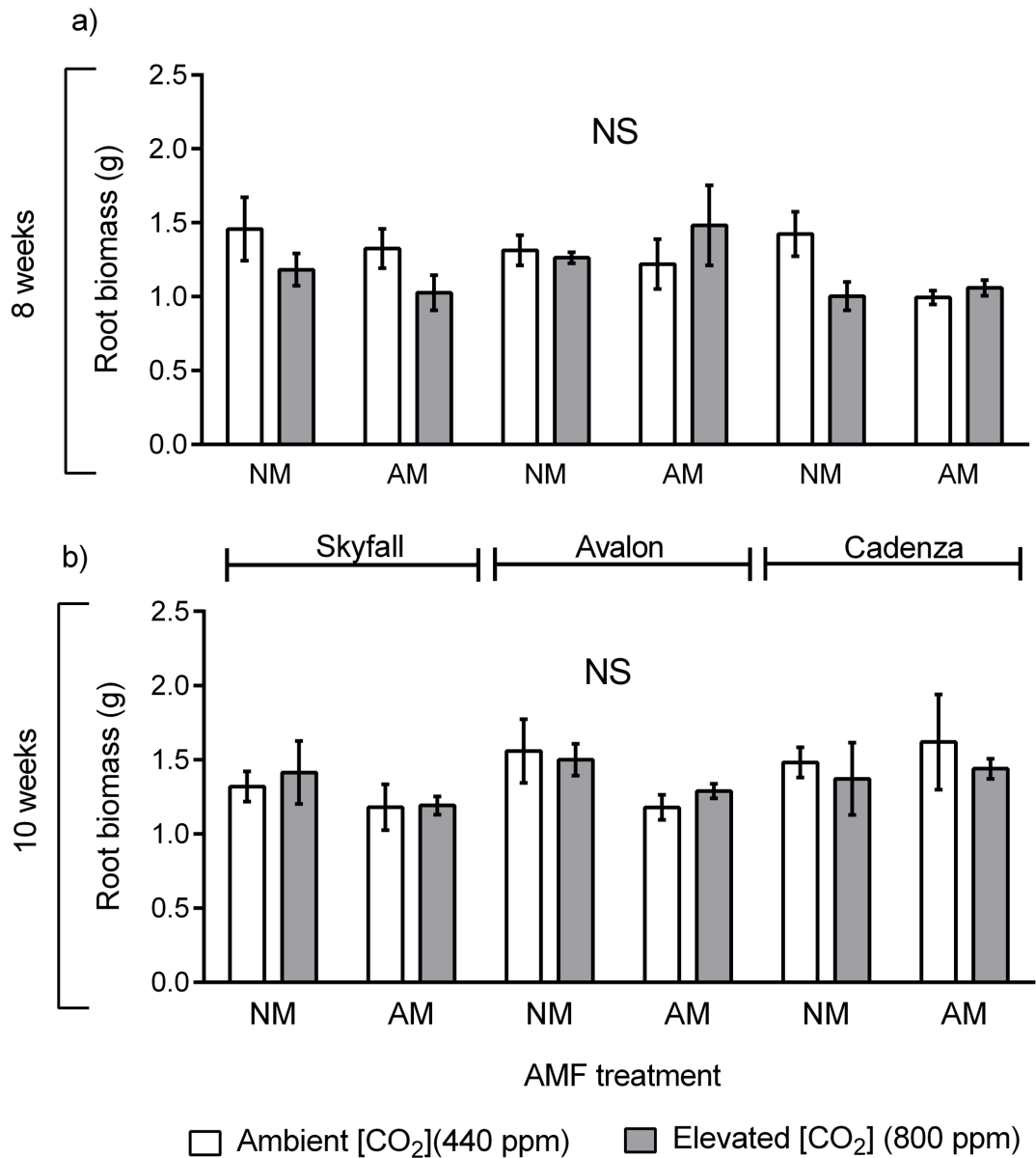


**Figure 4.3: Wheat shoot biomass with or without inoculation with *R. irregularis* at ambient and elevated atmospheric CO<sub>2</sub> concentrations ([CO<sub>2</sub>]).** shoot dry biomass: 8-weeks (a), 10-weeks (b). Experiments were conducted in three wheat cultivars, Skyfall, Avalon and Cadenza. Plants were inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (AM) or an autoclaved control (NM) and grown at ambient [CO<sub>2</sub>] (440 ppm -white bars) or elevated [CO<sub>2</sub>] (800 ppm – grey bars). Error bars represent the standard error of the mean. Different letters refer to significant differences (n=5, P<0.05, three-way ANOVA, TukeyHSD post-test) between treatments.

After ten weeks of growth, there was no significant interactions between [CO<sub>2</sub>], AMF and wheat cultivars in shoot biomass measurements. Elevated [CO<sub>2</sub>] had a positive effect on plant biomass ( $F=12.31$ ,  $d.f.=1,48$ ,  $P\leq.001$ ; three-way ANOVA, Table 4.2, Figure 4.3b). Mycorrhization generally reduced the wheat shoot biomass after 10 weeks of growth ( $F=5.18$ ,  $d.f.=1,48$ ,  $P=0.027$ ; three-way ANOVA, Table 4.2, Figure 4.3b). AMF colonisation led to 13% and 7% less shoot biomass at ambient and elevated [CO<sub>2</sub>] respectively in cv. Skyfall and 15% and 3% less at ambient and elevated [CO<sub>2</sub>] respectively in cv. Avalon. While in cv. Cadenza mycorrhization resulted in 2% more biomass at ambient [CO<sub>2</sub>] and 5% less at elevated [CO<sub>2</sub>]. However, at 10 weeks, there were no significant interactions between the treatments (Table 4.2).

There was no significant effect of [CO<sub>2</sub>] or AMF treatment on root biomass in any of the cultivars at either time point (Figure 4.4, Table 4.2).





**Figure 4.4: Wheat root biomass with or without inoculation with *R. irregularis* at ambient and elevated atmospheric CO<sub>2</sub> concentrations ([CO<sub>2</sub>]).** (a) 8 weeks, (b) 10 weeks. Experiments were conducted in three wheat cultivars, Skyfall, Avalon and Cadenza. Plants were inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (AM) or an autoclaved control (NM) and grown at ambient [CO<sub>2</sub>] (440 ppm -white bars) or elevated [CO<sub>2</sub>] (800 ppm – grey bars). Error bars represent the standard error of the mean. Different letters refer to significant differences (n=5,  $P < 0.05$ , three-way ANOVA, TukeyHSD post-test) between treatments, NS – no significant differences between means.

#### 4.4.1.3 Plant tissue phosphorus.

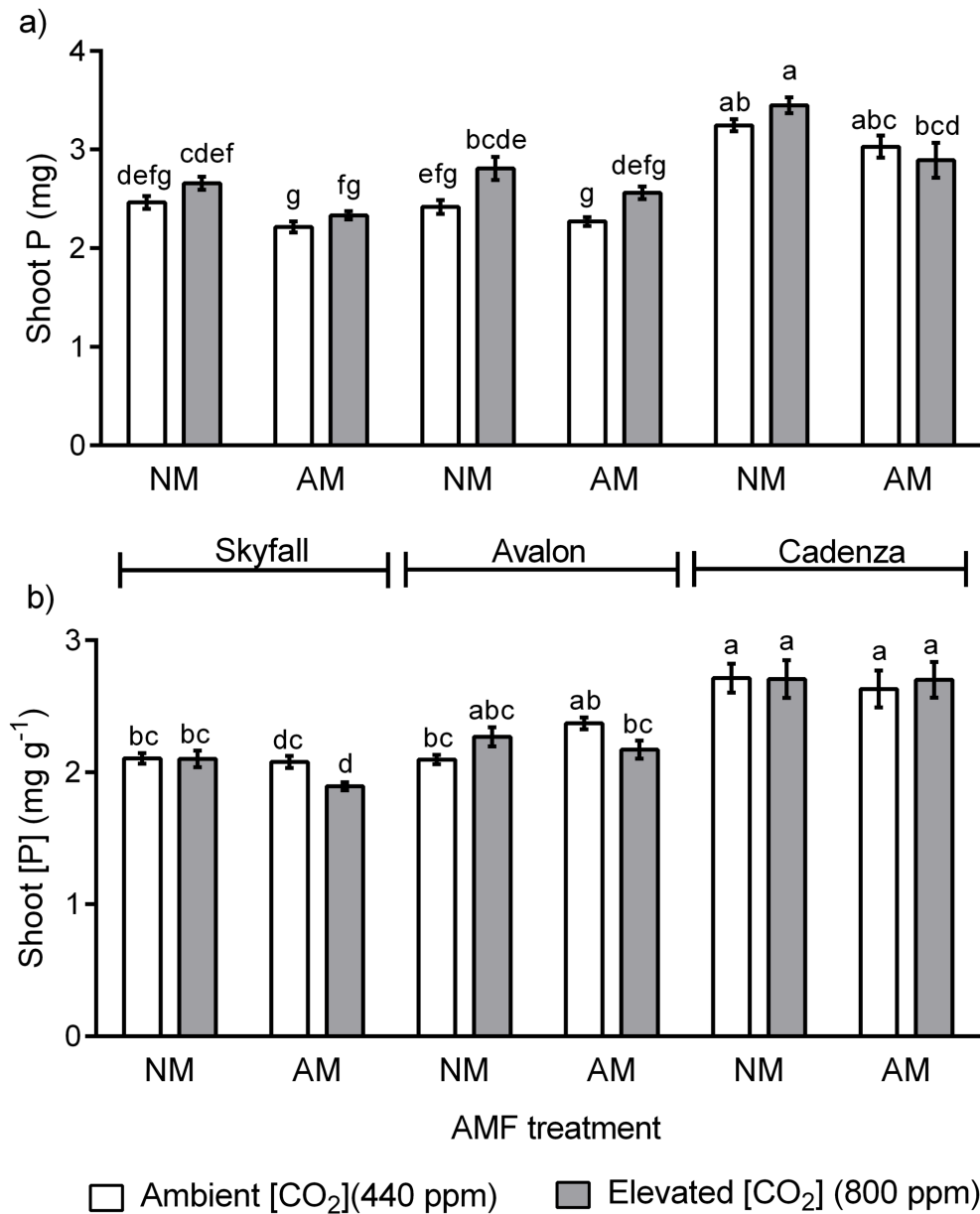
P within the shoot tissue, in terms of absolute quantities, was significantly less in the mycorrhizal compared to non-mycorrhizal plants ( $F=32.73$ ,  $d.f.=1,48$ ,  $P<0.001$ ; three-way ANOVA, Table 4.2, Figure 4.5a). In cv. Skyfall there was 10% and 12% less at ambient and elevated  $[CO_2]$  respectively, likewise in cv. Avalon, there was 6% and 9% less in total quantities of shoot P at ambient and elevated  $[CO_2]$  respectively. Finally, in cv. Cadenza at ambient  $[CO_2]$  shoot P was 7% lower, and at elevated  $[CO_2]$  it was 16% less in the mycorrhizal plants compared to the non-mycorrhizal plants.

Elevated  $[CO_2]$  had a positive effect on total quantities of P in the shoot tissue ( $F=12.11$ ,  $d.f.=1,48$ ,  $P=0.001$ ; three-way ANOVA, Table 4.2, Figure 4.5a). In the non-mycorrhizal treatment, there was 8% more total P in the plant tissue of cv. Skyfall in at elevated  $[CO_2]$  and in the mycorrhizal treatment there was 5% more. In cv. Avalon, there was 16% and 13% higher P at elevated  $[CO_2]$  in the non-mycorrhizal and mycorrhizal treatments respectively, whereas in cv. Cadenza total P was 6% more in the non-mycorrhizal treatment but 5% less in the mycorrhizal treatment at elevated  $[CO_2]$ . No significant interactions existed between any of the treatments in how they affected total P (Table 4.2).

When P was normalised to plant biomass, there was no overall significant effect of AMF treatment or  $[CO_2]$  treatment on shoot [P] (AMF:  $F=1.08$ ,  $d.f.=1,48$ ,  $P=0.305$ ; three-way ANOVA, Table 4.2, Figure 4.5b) ( $[CO_2]$ :  $F=2.16$ ,  $d.f.=1,48$ ,  $P=0.148$ ; three-way ANOVA, Table 4.2, Figure 4.5b).

[P] were determined by interactions between  $[CO_2]$  treatment, AMF treatment and wheat cultivar ( $[CO_2] \times AMF \times Cultivar$ :  $F=3.30$ ,  $d.f.=1,48$ ,  $P=0.045$ ; three-way ANOVA, Table 4.2, Figure 4.5b). For example, in cv. Skyfall and Cadenza there was no change in [P] between ambient and elevated  $[CO_2]$  in the non-mycorrhizal treatment, likely due to the similar increase in both biomass and total P within the tissue at elevated  $[CO_2]$  keeping the concentration stable. But, in the non-mycorrhizal treatment of cv. Avalon [P] were 8% higher at elevated  $[CO_2]$ . In the mycorrhizal plants, shoot [P] were lower at elevated  $[CO_2]$  in cv. Skyfall and Avalon by -9% and -8% respectively, whereas in cv. Cadenza, there was little difference (+3%). Therefore, the effect of elevated  $[CO_2]$  on shoot [P] was

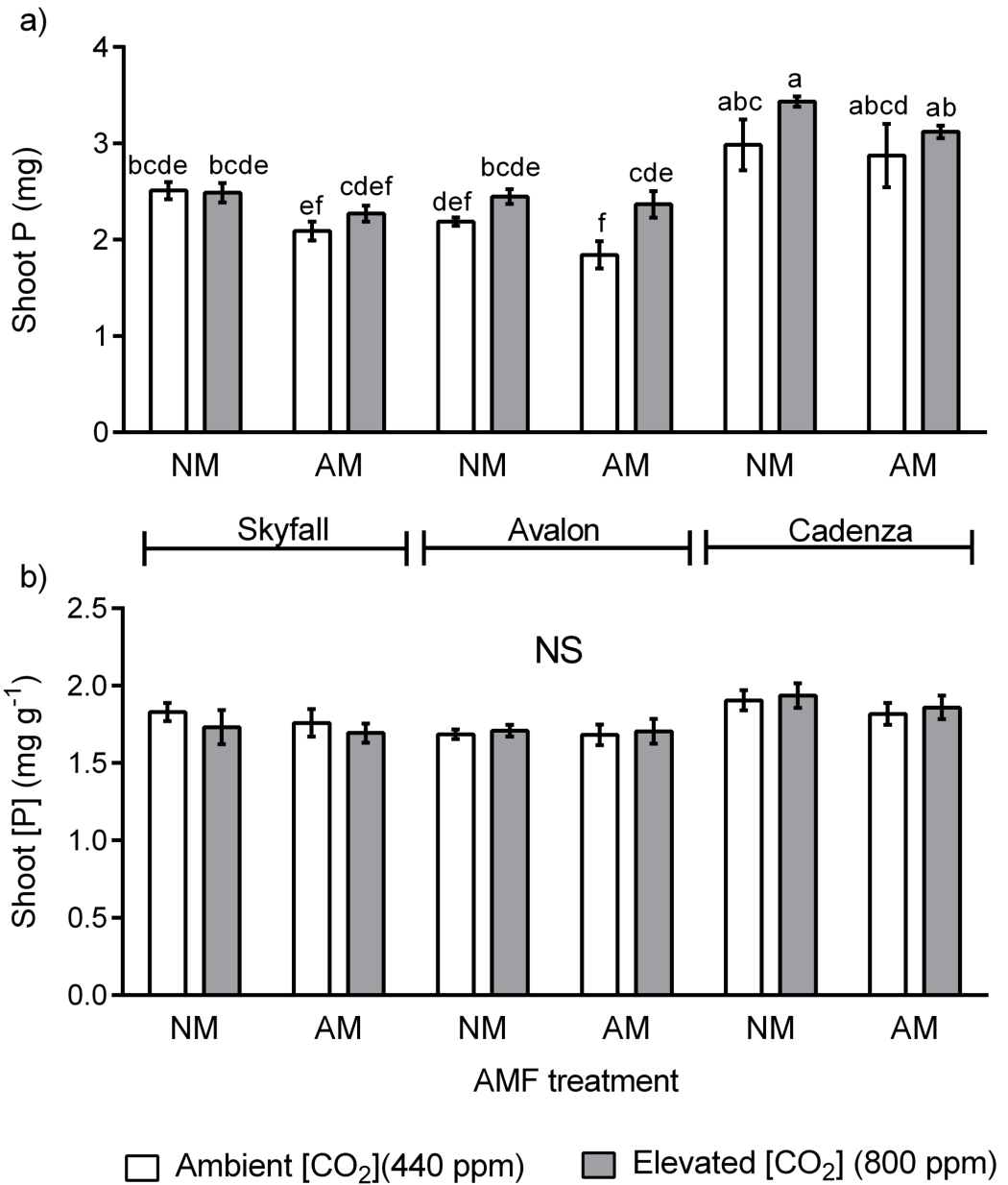
dependent on AMF treatment and differed between wheat cultivars ( $[\text{CO}_2] \times \text{AMF} \times \text{Cultivar}$ :  $F=3.30$ ,  $d.f.=1,48$ ,  $P=0.045$ ; three-way ANOVA, Table 4.2, Figure 4.5b).



**Figure 4.5: Shoot phosphorus assimilation by the host plant at eight weeks with or without inoculation with *R. irregularis* at ambient and elevated atmospheric CO<sub>2</sub> concentrations ([CO<sub>2</sub>]).** (a) absolute amounts, (b) concentrations. Experiments were conducted in three wheat cultivars, Skyfall, Avalon and Cadenza. Plants were inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (AM) or an autoclaved control (NM) and grown at ambient [CO<sub>2</sub>] (440 ppm -white bars) or elevated a[CO] (800 ppm – grey bars). Error bars represent the standard error of the mean. Different letters refer to significant differences ( $n=5$ ,  $P<0.05$ , three-way ANOVA, TukeyHSD post-test) between treatments.

When plants were grown for ten weeks, there were no significant differences in how AMF or [CO<sub>2</sub>] affected [P] in any of the wheat cultivars (Table 4.2). P within the shoot tissue in terms of absolute quantities was significantly lower in mycorrhizal plants compared to the non-mycorrhizal treatment at the ten week timepoint (  $F=11.91$ ,  $d.f.=1,48$ ,  $P=0.001$ ; three-way ANOVA, Table 4.2, Figure 4.6a). In mycorrhizal cv. Skyfall it was 17% and 9% less at ambient and elevated [CO<sub>2</sub>] respectively, likewise in mycorrhizal cv. Avalon, absolute quantities of shoot P were 16% and 3% lower at ambient and elevated [CO<sub>2</sub>] respectively. Finally, in mycorrhizal cv. Cadenza at ambient [CO<sub>2</sub>] shoot P was 4% less, and at elevated [CO<sub>2</sub>] it was 9% less compared to non-mycorrhizal counterparts.

Elevated [CO<sub>2</sub>] generally had a positive effect on total quantities of P in the shoot tissue at the ten week timepoint ( $F=14.57$ ,  $d.f.=1,48$ ,  $P<0.001$ ; three-way ANOVA, Table 4.2, Figure 4.6a). In the non-mycorrhizal treatment, there was no difference in total P in the plant tissue of cv. Skyfall at elevated [CO<sub>2</sub>] and in the mycorrhizal treatment total P 9% higher at elevated [CO<sub>2</sub>], compared to ambient [CO<sub>2</sub>]. In cv. Avalon, there was 12% and 28% more P at elevated [CO<sub>2</sub>] compared to ambient [CO<sub>2</sub>] in the non-mycorrhizal and mycorrhizal treatments respectively, whereas in cv. Cadenza total P was 15% higher in the non-mycorrhizal plants and 9% more in the mycorrhizal plants at elevated [CO<sub>2</sub>]. There were no significant interactions between any of the treatments in how they affected total P (Table 4.2).



**Figure 4.6: Shoot phosphorus assimilation by the host plant at ten weeks with or without inoculation with *R. irregularis* at ambient and elevated atmospheric CO<sub>2</sub> concentrations ([CO<sub>2</sub>]).** (a) absolute amounts, (b) concentrations. Experiments were conducted in three wheat cultivars, Skyfall, Avalon and Cadenza. Plants were inoculated with arbuscular mycorrhizal fungi inoculum containing *Rhizophagus irregularis* (AM) or an autoclaved control (NM) and grown at ambient [CO<sub>2</sub>] (440 ppm -white bars) or elevated [CO<sub>2</sub>] (800 ppm – grey bars). Error bars represent the standard error of the mean. Different letters refer to significant differences (n=5, P<0.05, three-way ANOVA, Tukey HSD post-test) between treatments. NS – no significant differences between means.

#### 4.4.2 Experiment 2: Carbon-for-nutrient exchange in symbionts under ambient [CO<sub>2</sub>] and elevated [CO<sub>2</sub>].

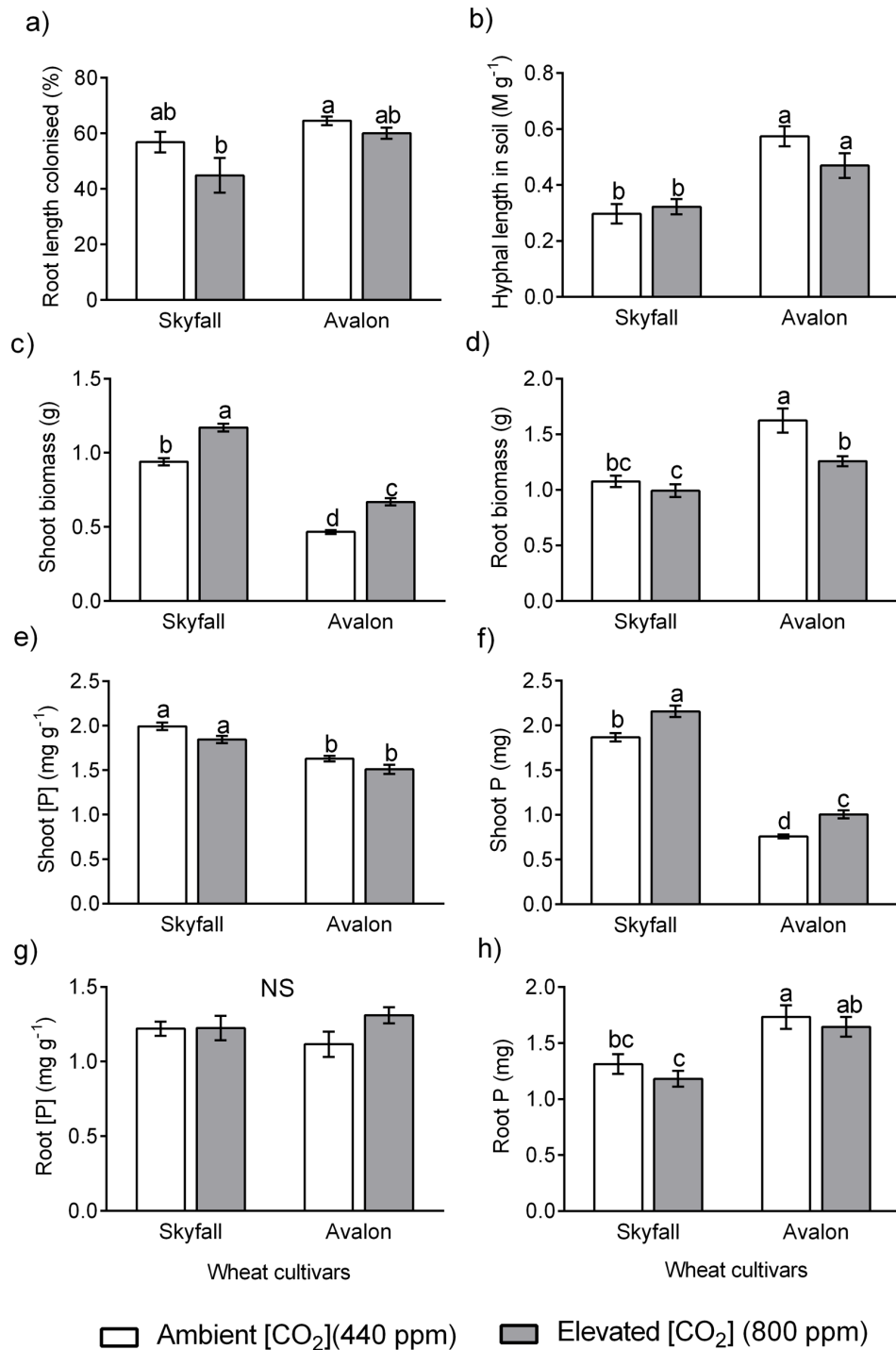
##### 4.4.2.1 Biomass, colonisation and total P.

Two cultivars Skyfall and Avalon were grown in association with a commercial AMF inoculant (*R. irregularis*), to assess how elevated [CO<sub>2</sub>] influenced carbon-for-nutrient exchange within the symbiosis. In contrast to experiment 1, there was no non-mycorrhizal treatment and only cv. Skyfall and Avalon were chosen for the isotope tracing experiments as in preliminary growth experiments they were more responsive to changes in [CO<sub>2</sub>] than cv. Cadenza. In this experiment elevated [CO<sub>2</sub>] had a significant effect on percentage root colonisation of AMF, with a small reduction in root colonisation occurring in both wheat cultivars (F=4.14, d.f.=1,44, P=0.048; two-way ANOVA, Table 4.3, Figure 4.7a). This corresponded to less arbuscule structures within the root system (F=3.49, d.f.=1,44, P=0.048; two-way ANOVA, Table 4.3), but there was no effect on vesicle structures (F=1.34, d.f.=1,44, P=0.283; two-way ANOVA, Table 4.3), or on the length of extraradical hyphae in the soils (F=0.78, d.f.=1,44, P=0.383; two-way ANOVA, Table 4.3, Figure 4.7b).

Similarly to experiment 1, elevated [CO<sub>2</sub>] had a positive effect on the shoot biomass of the mycorrhizal wheat plants, (25% cv. Skyfall, 43% cv. Avalon) (F=88.8, d.f.=1,44, P<0.001; two-way ANOVA, Table 4.3, Figure 4.7c). However, root biomass was lower under elevated [CO<sub>2</sub>] conditions (-8% cv. Skyfall, -23% cv. Avalon) (F=8.53, d.f.=1,44, P=0.005; two-way ANOVA, Table 4.3, Figure 4.7d). As shoot biomass was higher at elevated [CO<sub>2</sub>] absolute levels of P were also significantly more by 16% and 32% in cv. Skyfall and Avalon respectively (F=33.2, d.f.=1,44, P<0.001; two-way ANOVA, Table 4.3, Figure 4.7f). However, concentrations of P in the shoot tissue were 7% less in both cultivars under elevated [CO<sub>2</sub>] (F=10.1, d.f.=1,44, P=0.003; two-way ANOVA, Table 4.3, Figure 4.7e). [CO<sub>2</sub>] had no significant effect on absolute levels or concentrations of P in the root tissue of either cultivar (Root absolute P: F=1.52, d.f.=1,44, P=0.224; two-way ANOVA, Table 4.3, Figure 4.7h), (Root concentrations of P: F=2.49, d.f.=1,44, P=0.122; two-way ANOVA, Table 4.3, Figure 4.7g).

**Table 4.3:** Summary of two-way ANOVA (GLM) results testing the effects of [CO<sub>2</sub>] concentrations on the root colonisation of three wheat cultivars inoculated with *R. irregularis*. Significant values highlighted in bold.

<b>Variable</b>	<b>Wheat cultivar</b>		<b>[CO<sub>2</sub>] treatment</b>		<b>[CO<sub>2</sub>] x Cultivar</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	<i>1, 44</i>	<i>d.f</i>	<i>1,44</i>	<i>d.f</i>	<i>1,44</i>
Shoot biomass (g)	452.0	<b>&lt;0.001</b>	88.8	<b>&lt;0.001</b>	0.39	0.535
Root biomass (g)	37.24	<b>&lt;0.001</b>	8.53	<b>0.005</b>	1.22	0.275
Root colonisation (%)	7.76	<b>0.008</b>	4.14	<b>0.048</b>	0.31	0.580
ER hyphae length (m g <sup>-1</sup> )	34.43	<b>&lt;0.001</b>	0.78	0.383	3.38	0.073
Arbuscules (%)	1.33	0.261	3.49	<b>0.048</b>	1.19	0.322
Vesicles (%)	0.32	0.579	1.34	0.283	1.08	0.356
Shoot [P] (mg g <sup>-1</sup> )	69.22	<b>&lt;0.001</b>	10.1	<b>0.003</b>	0.11	0.740
Shoot P (mg)	588.3	<b>&lt;0.001</b>	33.2	<b>&lt;0.001</b>	0.22	0.642
Root [P] (mg g <sup>-1</sup> )	0.00	0.970	2.49	0.122	1.45	0.234
Root P (mg)	24.66	<b>&lt;0.001</b>	1.52	0.224	0.06	0.803



**Figure 4.7: The effect of ambient and elevated atmospheric CO<sub>2</sub> concentrations ([CO<sub>2</sub>]) on wheat biomass, tissue phosphorus and AMF colonisation.** (a) Root length colonised by arbuscular mycorrhizal fungi (%), (b) AMF extra-radical hyphal lengths in the soil, (c) tissue phosphorus: shoot absolute amounts, (d) shoot concentrations, (e) root absolute amounts, (f) root concentrations, (g) shoot biomass, (h) root biomass. Experiments were conducted in two wheat cultivars, Skyfall, and Avalon. Plants were inoculated with *Rhizophagus irregularis* and grown at ambient [CO<sub>2</sub>] (440 ppm -white bars) or elevated [CO<sub>2</sub>] (800 ppm – grey bars). Error bars represent the standard error of the mean. Different letters refer to significant



differences (n=12,  $P < 0.05$ , two-way ANOVA, TukeyHSD post-test) between treatments. NS – no significant differences between means.

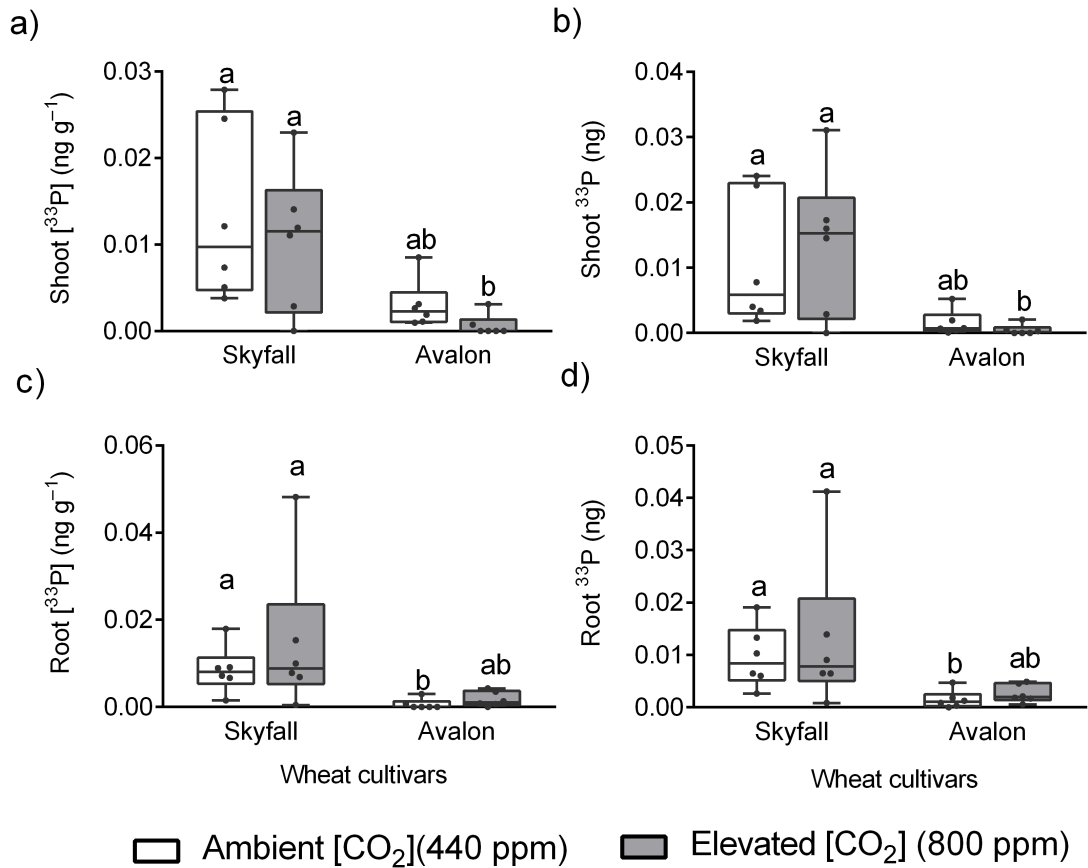
#### 4.4.2.2 $^{33}\text{P}$ transfer from fungus-to-plant.

There was  $^{33}\text{P}$  transfer from the mycorrhizal partner to the wheat shoots; however, cv. Avalon gained significantly less  $^{33}\text{P}$  through its mycorrhizal partner than cv. Skyfall at elevated  $[\text{CO}_2]$ . In cv. Skyfall shoot material at elevated  $[\text{CO}_2]$ , AMF acquired 4.9% of the total  $^{33}\text{P}$  added to the pots whereas, in cv. Avalon AMF acquired only 0.3% of total  $^{33}\text{P}$  supplied to the pot. Within each cultivar there was no significant difference in the amount of mycorrhizal acquired  $^{33}\text{P}$  in the shoots under elevated or ambient  $[\text{CO}_2]$  conditions; either in terms of absolute quantities or when normalised to plant biomass (shoot absolute  $^{33}\text{P}$ :  $H=11.43$ , d.f.=3,  $P=0.01$ , Kruskal-Wallis, Table 4.4, Figure 4.8b) (shoot  $^{33}\text{P}$ :  $H=13.03$ , d.f.=3,  $P=0.005$ , Kruskal-Wallis, Table 4.4, Figure 4.8a).

**Table 4.4:** Summary of Kruskal-Wallis results testing the effects of  $[\text{CO}_2]$  concentrations on the carbon-for-nutrient exchange between two wheat cultivars and *R. irregularis*. Significant values highlighted in bold.

Variable	Kruskal-Wallis	
	H	P
Degrees of freedom	d.f	3
Shoot $^{33}\text{P}$ (ng g <sup>-1</sup> )	13.03	<b>0.005</b>
Shoot $^{33}\text{P}$ (ng)	11.43	<b>0.01</b>
Root $^{33}\text{P}$ (ng g <sup>-1</sup> )	13.78	<b>0.003</b>
Root $^{33}\text{P}$ (ng)	12.69	<b>0.005</b>

cv. Avalon also seemed to accumulate less mycorrhizal acquired  $^{33}\text{P}$  in the root material, receiving significantly less than cv. Skyfall at ambient  $[\text{CO}_2]$  (root absolute  $^{33}\text{P}$ :  $H=12.69$ , d.f.=3,  $P=0.005$ , Kruskal-Wallis, Table 4.4, Figure 4.8d) (root  $^{33}\text{P}$ :  $H=13.78$ , d.f.=3,  $P=0.003$ , Kruskal-Wallis, Table 4.4, Figure 4.8c). cv. Skyfall acquired 3.5% of supplied  $^{33}\text{P}$  while cv. Avalon gained only 0.8%. Rising  $[\text{CO}_2]$  had no significant effect on mycorrhizal acquired  $^{33}\text{P}$  in the root material of either cultivar for both absolute quantities or when normalised to plant biomass (see Figure 4.8c,d)

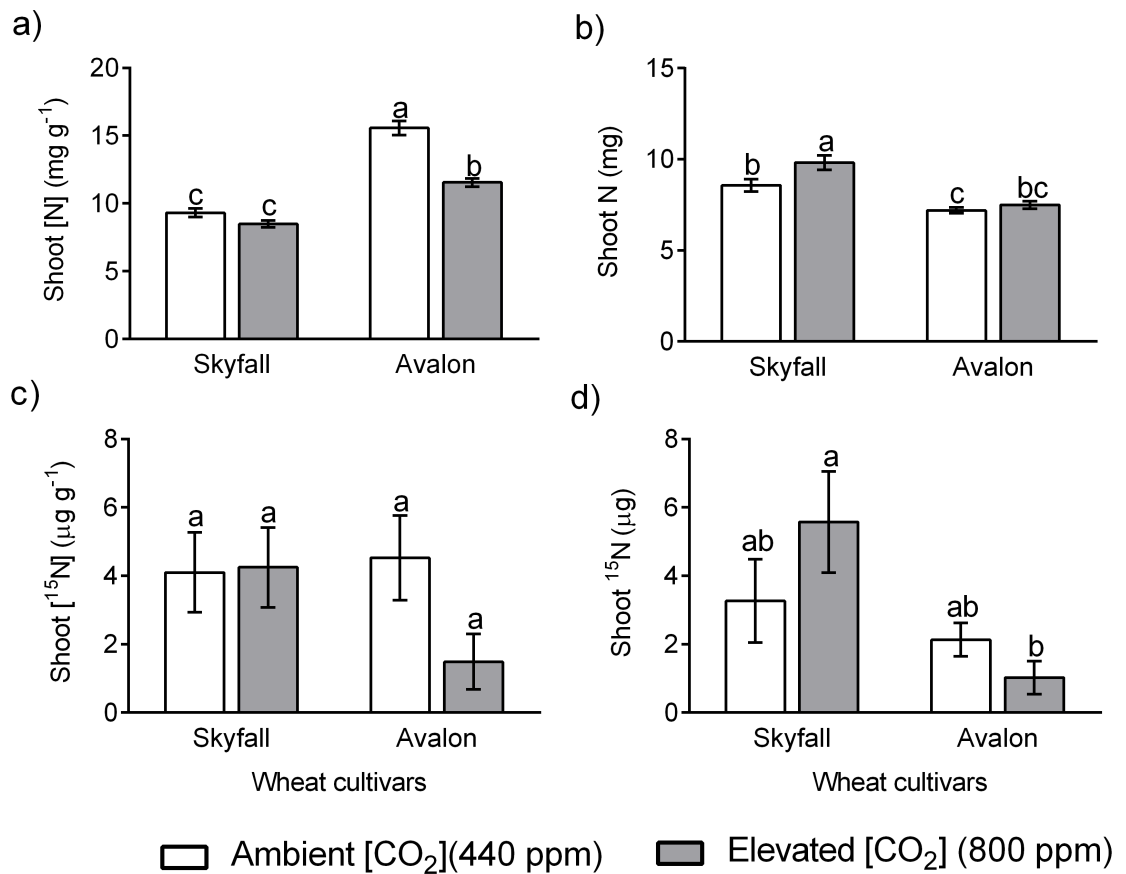


**Figure 4.8: Mycorrhizal acquired  $^{33}\text{P}$  in wheat tissue at ambient and elevated atmospheric  $\text{CO}_2$  concentrations.** Shoot plant tissue: (a) concentrations, (b) absolute amounts. Root tissue: (c) concentrations, (d) absolute amounts. Experiments were conducted in two wheat cultivars, Skyfall, and Avalon. Plants were inoculated with *Rhizophagus irregularis* and grown at ambient  $[\text{CO}_2]$  (440 ppm -white bars) or elevated  $[\text{CO}_2]$  (800 ppm – grey bars). Different letters refer to significant differences ( $n=6$ ,  $P<0.05$ , Kruskal-Wallis, Dunn’s post-test) between treatments.

#### 4.4.2.3 Nitrogen concentrations and $^{15}\text{N}$ transfer from fungus-to-plant.

Plant tissue N was generally higher under elevated  $[\text{CO}_2]$  conditions, in cv. Skyfall total N was up to 15% more, whereas in cv. Avalon it was only 4% higher ( $F=7.13$ ,  $d.f.=2,36$ ,  $P=0.011$ ; two-way ANOVA, Table 4.5, Figure 4.9b). When N was normalised to plant biomass, there was a significant interaction between  $[\text{CO}_2]$  and wheat cultivar ( $[\text{CO}_2] \times \text{cultivar } F=18.9$ ,  $d.f.=2,36$ ,  $P<0.001$ ; two-way ANOVA, Table 4.5, Figure 4.9a), with the concentration of N significantly less in cv. Avalon (-26%) and marginally less in cv. Skyfall (-9%) ( $[\text{CO}_2]$  :  $F=43.0$ ,  $d.f.=2,36$ ,  $P<0.001$ ; two-way ANOVA, Table 4.5, Figure 4.9a).

$^{15}\text{N}$  transfer from the mycorrhizal partner to the wheat shoots was observed. Still, there were no significant differences in amounts mycorrhizal acquired  $^{15}\text{N}$  in the shoots of plants grown under elevated  $[\text{CO}_2]$  or ambient  $[\text{CO}_2]$  conditions either in terms of absolute quantities or when normalised to plant biomass (absolute  $^{15}\text{N}$ :  $F=0.00$ ,  $d.f.=2,36$ ,  $P=0.974$ ; two-way ANOVA, Table 4.5, Figure 4.9d), ( $^{15}\text{N}$ ):  $F=1.69$ ,  $d.f.=2,36$ ,  $P=0.208$ ; two-way ANOVA, Table 4.5, Figure 4.9c). However, cv. Avalon seemed to gain less absolute  $^{15}\text{N}$  through its mycorrhizal partner than cv. Skyfall at elevated  $[\text{CO}_2]$ .



**Figure 4.9: Shoot tissue nitrogen and mycorrhizal acquired  $^{15}\text{N}$  at ambient and elevated atmospheric  $\text{CO}_2$  concentrations ( $[\text{CO}_2]$ ).** Nitrogen ( $n=12$ ): (a) concentrations, (b) absolute amounts.  $^{15}\text{N}$  ( $n=6$ ): (c) concentrations, (d) absolute amounts. Experiments were conducted in two wheat cultivars, Skyfall, and Avalon. Plants were inoculated with *Rhizophagus irregularis* and grown at ambient  $[\text{CO}_2]$  (440 ppm -white bars) or elevated  $[\text{CO}_2]$  (800 ppm – grey bars). Error bars represent the standard error of the mean. Different letters refer to significant differences ( $P < 0.05$ , two-way ANOVA, TukeyHSD post-test)

**Table 4.5:** Summary of two-way ANOVA (GLM) results testing the effects of [CO<sub>2</sub>] concentrations on plant tissue nitrogen and mycorrhizal acquired <sup>15</sup>N of wheat cultivars inoculated with *R. irregularis*. Significant values highlighted in bold, where interactions were significant main effects were not reported.

<b>Variable</b>	<b>Wheat cultivar</b>		<b>[CO<sub>2</sub>] treatment</b>		<b>[CO<sub>2</sub>] Cultivar x</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	1, 36	<i>d.f</i>	2,36	<i>d.f</i>	2,36
Shoot [N] (mg g <sup>-1</sup> )	159.2	<0.001	43.0	<0.001	18.9	<b>&lt;0.001</b>
Shoot N (mg)	41.4	<b>&lt;0.001</b>	7.13	<b>0.011</b>	2.73	0.107
<i>Degrees of freedom</i>	<i>d.f.</i>	1, 20	<i>d.f.</i>	1,20	<i>d.f.</i>	1,20
Shoot [ <sup>15</sup> N] (μg g <sup>-1</sup> )	1.1	0.306	1.69	0.208	2.06	0.167
Shoot <sup>15</sup> N (μg)	6.05	<b>0.023</b>	0.00	0.974	4.08	0.057

#### 4.4.2.4 Carbon allocation from plant-to-fungus.

The concentration of C assimilated in the shoot material was higher when plants were exposed to elevated [CO<sub>2</sub>], by 38% and 57% in cv. Avalon and Skyfall respectively (shoot [C] μg g<sup>-1</sup>: F=72.81, d.f.=1,44, P<0.001; two-way ANOVA; Table 4.7, Figure 4.10a). C assimilated in the root material, also significantly higher at elevated [CO<sub>2</sub>] by 60% and 53% in cv. Avalon and Skyfall respectively (root [C] μg g<sup>-1</sup>: F=25.5, d.f.=1,44, P<0.001; two-way ANOVA; Table 4.7, Figure 4.10b).

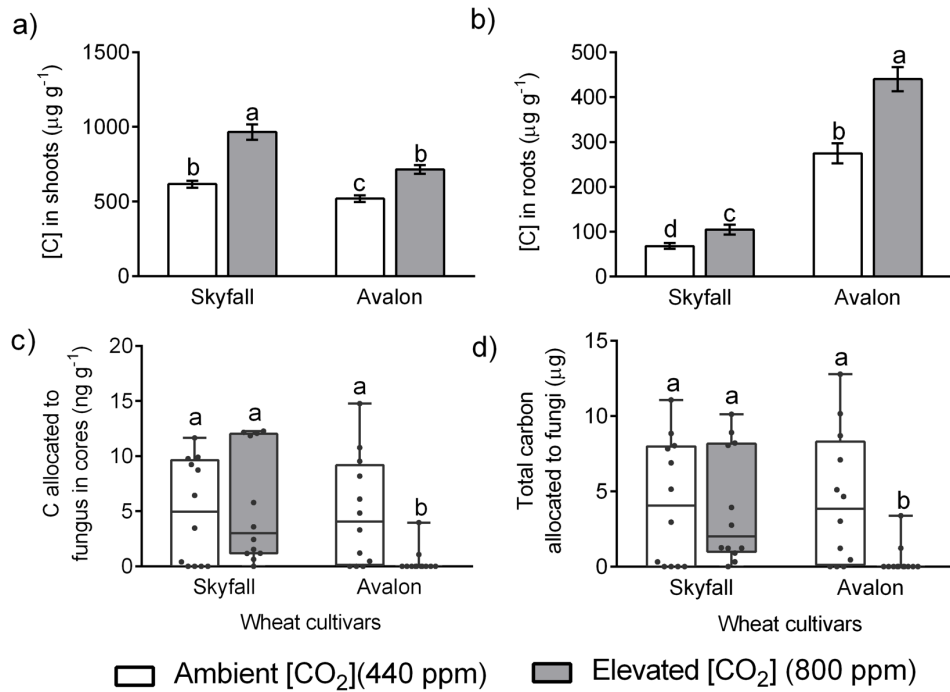
C transfer to mycorrhizal fungi within the core, or when scaled up to the whole pot, was not higher at elevated [CO<sub>2</sub>] in cv. Skyfall. However, in cv. Avalon there was significantly less C allocated to the fungal partner at elevated [CO<sub>2</sub>] (C allocated to the fungi in the core: H=14.61, d.f.=3, P=0.002, Kruskal-Wallis, Table 4.6, Figure 4.10c), (C allocated to the fungi in the pot: H=13.41, d.f.=3, P=0.004, Kruskal-Wallis, Table 4.6, Figure 4.10d). Nevertheless, only a small amount of C fixed by the plant passes to the fungal partner over a 16h photoperiod, over 90% of the <sup>14</sup>C fixed remains in the plant material, and >1% is in the extraradical fungal mycelium.

**Table 4.6:** Summary of Kruskal-Wallis results testing the effects of [CO<sub>2</sub>] concentrations on the carbon-for-nutrient exchange between two wheat cultivars and *R. irregularis*. Significant values highlighted in bold.

<b>Variable</b>	<b>Kruskal-Wallis</b>	
	<b>H</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	3
C allocation to fungi in cores (ng g <sup>-1</sup> )	14.61	<b>0.002</b>
C allocation to fungi in the pot (μg)	13.41	<b>0.004</b>

**Table 4.7:** Summary of two-way ANOVA (GLM) results testing the effects of [CO<sub>2</sub>] on C assimilation in the plant tissue of wheat cultivars inoculated with *R. irregularis*. Significant values highlighted in bold.

<b>Variable</b>	<b>Wheat cultivar</b>		<b>[CO<sub>2</sub>] treatment</b>		<b>[CO<sub>2</sub>] x Cultivar</b>	
	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>	<b>F</b>	<b>P</b>
<i>Degrees of freedom</i>	<i>d.f</i>	1, 44	<i>d.f</i>	1,44	<i>d.f</i>	1,44
C assimilation in the shoots (μg g <sup>-1</sup> )	26.15	<b>&lt;0.001</b>	72.81	<b>&lt;0.001</b>	0.66	0.420
C assimilation in the roots (μg g <sup>-1</sup> )	260.7	<b>&lt;0.001</b>	25.5	<b>&lt;0.001</b>	0.19	0.664



**Figure 4.10: Carbon assimilated in the plant shoot and root material and transferred to fungal partners over one photoperiod at ambient and elevated atmospheric CO<sub>2</sub> concentrations.** Plant tissue carbon (C) assimilation (n=12): (a) Shoot concentration, (b) root concentration, (c) C allocation to the fungus in cores (n=6), (d) C allocation to the fungus in the pot (n=6). Experiments were conducted in two wheat cultivars, Skyfall, and Avalon. Plants were inoculated with *Rhizophagus irregularis* and grown at ambient [CO<sub>2</sub>] (440 ppm -white bars) or elevated [CO<sub>2</sub>] (800 ppm – grey bars). Error bars represent the standard error of the mean. Different letters refer to significant differences. (a, b)  $P < 0.05$ , two-way ANOVA, TukeyHSD post-test, (c, d)  $P < 0.05$ , Kruskal-Wallis, Dunn’s Multiple Comparison Test, post-test.

## **4.5 Discussion**

### **4.5.1 Experiment 1: The interaction between [CO<sub>2</sub>] and wheat-AMF symbiosis.**

#### **4.5.1.1 The effect of [CO<sub>2</sub>] and AMF on wheat biomass**

Overall, elevated [CO<sub>2</sub>] had a positive effect on shoot biomass, and the mycorrhizal plants had a lower biomass than their non-mycorrhizal counterparts. However, at the eight week timepoint the extent of the above trends was highly dependent on treatment interactions and differences between the wheat cultivars. Within cv. Skyfall and Avalon mycorrhizal plants had amplified biomass gains under elevated [CO<sub>2</sub>] compared to non-mycorrhizal plants, but this trend was not present in cv. Cadenza. Additionally, within cv. Skyfall and Avalon the biomass differences between mycorrhizal plants and non-mycorrhizal plants was more substantially at ambient [CO<sub>2</sub>] than at elevated [CO<sub>2</sub>]. However, cv. Cadenza showed the opposite pattern with mycorrhizal plants having 4% lower biomass in ambient [CO<sub>2</sub>] and 13% less in elevated [CO<sub>2</sub>] when compared to non-mycorrhizal plants. The interactions between treatments did not persist to the ten week time point, with only the main effects of the treatments remaining significant. Root biomass was not altered by either [CO<sub>2</sub>] or AMF treatments at either timepoint. Due to the combination of low nutrient availability (through a sand and perlite substrate and minimal fertiliser regime) and elevated [CO<sub>2</sub>] in this experiment, enhancement of root growth traits was expected as plants under limited nutrient conditions are predicted to invest more highly in mechanisms of acquiring nutrients such as root material; but, elevated [CO<sub>2</sub>] did not affect root biomass (Figure 4.4).

The effects of [CO<sub>2</sub>] on root biomass can be highly variable between studies, with many not showing the expected enhancement of root growth (Including chickpea and field pea) (Gavito et al., 2000; Jin et al., 2015; Pandey et al., 2015a). Some studies even showed a reduction in root-to-shoot growth at high [CO<sub>2</sub>], for example, in cotton, white lupin (Campbell & Sage, 2002) and Ponderosa pine (Walker et al., 1995). Species that form proteoid roots can react to nutrient limitations through proteoid root production rather than increasing total root system biomass, this is advantageous as proteoid roots are less expensive in terms of C and energy and therefore are a more efficient means of gaining

nutrients (Gardner et al., 1981; Campbell & Sage, 2002). Similarly, other plants may utilise their relationships with AMF, investing additional C from high [CO<sub>2</sub>] conditions to enhance AMF biomass rather than their root system biomass (BassiriRad et al., 2001). However, evidence from experiment 2 within this chapter suggests these wheat cultivars allocated no more carbon to AMF partners at elevated [CO<sub>2</sub>]. Alternatively, [CO<sub>2</sub>] has been shown to increase plant WUE and may lead to a higher soil moisture content which will enhance the diffusion of nutrients within the soil towards the roots and allow higher nutrient uptake without increased investment in the root system (Barracough & Tinker, 1981).

The present experiments demonstrate that elevated [CO<sub>2</sub>] has a positive effect on shoot biomass at the two time-points sampled (eight and ten weeks) (Figure 4.3). These results are consistent with the consensus that C3 crops such as wheat respond positively to increasing [CO<sub>2</sub>] (Mitchell et al., 1993; Tubiello et al., 1999; Zhu et al., 2016). However, higher plant growth can only be maintained over extended periods if nutrients or water availability is not limiting (Drake et al., 1997; Pandey et al., 2015b).

In this experiment, wheat plants were grown in low nutrient sand and perlite substrate, which may have resulted in the plants being unable to fully take advantage of the higher [CO<sub>2</sub>] levels due to nutrient deficiencies (Mitchell et al., 1993). The low plant P and N uptake measured within this chapter compared to Chapter 2, where the plants were grown in an agricultural soil substrate, support that the plants had low nutrient availability. Dependent on the cultivar the N values were 2-3 times higher in Chapter 2, and the P values were 2-6 times higher in Chapter 2. Nutrient limitations often restrict plant growth responses to rising [CO<sub>2</sub>], which has led to the suggestion that symbiotic fungi may play a role in enhancing plant responses to increasing [CO<sub>2</sub>], through supplementing plant nutrient uptake (Cavagnaro et al., 2011; Pandey et al., 2015b). However, AMF inoculation did not enhance P uptake at ambient or elevated [CO<sub>2</sub>].

Despite this, the plant growth response to elevated [CO<sub>2</sub>] was more substantial in mycorrhizal wheat cv. Skyfall and cv. Avalon, resulting in a 16% increase in biomass at elevated [CO<sub>2</sub>] compared to an 8% at ambient [CO<sub>2</sub>] in cv. Skyfall and a 23% increase in biomass versus a 7% increase in biomass in cv. Avalon. While the increased 'carbon fertilisation' effect over non-mycorrhizal plants



cannot be attributed directly to alleviation of P limitations within the experiment, the AMF may be supplying other essential nutrients, for example, N. This is consistent with results in *P. sativum* L. where AMF increased plant biomass at elevated [CO<sub>2</sub>] despite no increase total P content associated with AMF. However, AMF were able to increase plant tissue N over non-mycorrhizal plants (Gavito et al., 2000). Plants within this experiment were able to gain N from an AMF partner (as shown in experiment 2), however N supply via AMF was not enhanced by elevated [CO<sub>2</sub>], and within cv. Avalon [N] within the plant decreased at elevated [CO<sub>2</sub>]. Therefore, enhanced N uptake through AMF associations is unlikely to explain the 'carbon fertilisation' effect seen in mycorrhizal plants.

On the other hand, AMF have been shown to increase nutrient use efficiency (NUE) in some plants in some cases (Gavito et al., 2000; Zhu et al., 2016). Therefore, mycorrhizal plants may be able to increase their NUE more effectively at elevated [CO<sub>2</sub>], leading to greater plant growth responses regardless of no additional P or N uptake (Gavito et al., 2000). However, other studies found no such effect of AMF on NUE (Jakobsen et al., 2016).

Nevertheless, despite the increased growth response at elevated [CO<sub>2</sub>] in mycorrhizal plants, they never surpassed non-mycorrhizal plants in shoot biomass measurements. This was potentially due to the AMF associations themselves, resulting in a negative plant growth response. Two hypotheses prevail on why AMF may result in a plant growth penalty compared to non-mycorrhizal plants. Firstly, AMF may act as a large C drain on the plant (Reynolds et al., 2005). However, AMF are unlikely to be acting a large carbon drain in this case, as when C allocation from the plant to the fungus was measured it represented >1% of the plants C budget. Secondly, that colonisation by AMF results in a downregulation of the plant's own P uptake pathway and therefore if the symbiosis is not functional can lead to a decrease in overall plant nutrient uptake (Smith et al., 2011). AMF may be triggering a decrease in the plants direct-uptake pathway for phosphorus, as results show P uptake within the wheat cultivars was reduced in mycorrhizal plants.

In cv. Skyfall and Avalon, the reduction in biomass in response to AMF, is less under high [CO<sub>2</sub>] conditions. It is possible that enhanced plant C fixation under elevated [CO<sub>2</sub>], due to increased efficiency of C3 photosynthesis within the wheat plants, could balance any cost associated with the symbiosis. Other than direct

carbon drain to the fungus, AMF association could be costly due to temporary costly defence responses they elicit within plant partners (Fernández et al., 2019). Negative plant growth responses to AMF found at high P fertilisation levels under ambient [CO<sub>2</sub>] were mitigated at elevated [CO<sub>2</sub>] in *Brachypodium distachyon*, suggesting any C costs to the plant was compensated by increased C assimilation at elevated [CO<sub>2</sub>] (Jakobsen et al., 2016).

Plant responses to AMF and atmospheric [CO<sub>2</sub>] remained similar between the eight and ten weeks timepoints. There was no evidence that plants were acclimating to elevated [CO<sub>2</sub>] as the difference in biomass between plants grown at ambient and elevated [CO<sub>2</sub>] increased between eight and ten weeks. Equally, AMF did not seem to become more beneficial to the plant cultivars over time.

#### **4.5.1.2 The effect of [CO<sub>2</sub>] and AMF on wheat tissue phosphorus**

Absolute quantities of P in the shoot material generally increased with elevated [CO<sub>2</sub>], in line with plant biomass increases (Figure 4.5), therefore, maintaining [P] between ambient and elevated [CO<sub>2</sub>]. Facilitating the growth of larger wheat plants at elevated [CO<sub>2</sub>], which inherently draw up more nutrients from the soil, will rely on increasing chemical fertiliser inputs if more sustainable alternatives to aid plant nutrient uptake such as AMF inoculants are not viable.

However, AMF seemed unable to enhance plant nutrient uptake at all, in fact, absolute quantities of P within the shoot material decreased in the presence of AMF when compared to non-mycorrhizal control plants at both the eight and ten week time points. Reduced P uptake in mycorrhizal plants is often associated with a “switching off” of the direct plant P uptake pathway in response to AMF colonisation (Javot et al., 2007; Smith et al., 2011), which is not effectively replaced by the mycorrhizal uptake pathway and has been observed on many occasions, including in wheat plants (Li et al., 2008; Smith et al., 2009, 2011). The only way of directly assessing differences in mycorrhizal nutrient uptake at different [CO<sub>2</sub>] is through tracing transfer of P and N from fungi to plants.

#### **4.5.2 Mycorrhizal carbon-for-nutrient exchange**

##### **4.5.2.1 AMF colonisation and extra-radical hyphal lengths.**

In the isotope tracing experiment, there was a small decrease in percentage root length colonisation overall between ambient and elevated [CO<sub>2</sub>]. However, there

were no significant differences between ambient and elevated [CO<sub>2</sub>] within the cultivars when the Tukey post-test was performed. A decrease in mycorrhizal colonisation following an increase in [CO<sub>2</sub>] goes against meta-analyses trends (Staddon & Fitter, 1998; Fitter et al., 2000; Treseder, 2004; Alberton et al., 2005), however it is not unprecedented as decreases in colonisation have been observed in a small number of previous experiments, and no change is commonly observed.

The decrease in AMF colonisation could not be accounted for by a dilution effect within changing root biomass as root biomass generally decreased with elevated [CO<sub>2</sub>]. Extra-radical hyphal lengths were not altered by rising [CO<sub>2</sub>]. However, there were cultivars differences with cv. Avalon's extra-radical hyphae averaging at 69% longer than cv. Skyfall (Figure 4.7). These cultivar differences are likely related to differences in root biomass allocation between the cultivars as cv. Avalon has a much higher biomass allocation to the root system (Staddon et al., 1999; Gavito et al., 2002).

Despite no noted increases in fungal biomass with elevated [CO<sub>2</sub>], they could still be providing enhanced nutrient uptake to the plant partner if the symbiosis is more functional at elevated [CO<sub>2</sub>]. Measuring the presence of fungal structures has been shown many times not to correlate well with the functionality of the symbiosis in terms of fungal acquired nutrients (Sanders et al., 1998), as was shown within Chapter 2, where a large increase in root colonisation after inoculation resulted in no more AM-acquired nutrients. AMF functioning can be highly context-dependent and be driven, among other things, by fungal ID and environmental factors (Munkvold et al., 2004; Hoeksema et al., 2010; Field & Pressel, 2018). Therefore, it is not unlikely that higher levels of [CO<sub>2</sub>], which can have a strong impact on plant physiology and the environment such as reducing stomatal conductance and enhancing soil moisture conditions could impact AMF functioning. However, within this experiment, no increase in AMF functioning was observed at elevated [CO<sub>2</sub>].

#### **4.5.2.2 Plant carbon assimilation and carbon allocation to the fungus.**

As expected, the C assimilated in the plant material was much higher at elevated [CO<sub>2</sub>] by an average of 48% in the shoot tissue and 57% in the root tissue (Figure 4.10). Enhanced C assimilation supports the theory that rising [CO<sub>2</sub>] increases photosynthesis leading to the growth in plant biomass observed at elevated [CO<sub>2</sub>]

in this experiment (25% cv. Skyfall, 43% cv. Avalon) (Figure 4.7). Previously, plants have been shown to invest higher amounts of C below ground as [CO<sub>2</sub>] increases (Drigo et al., 2010, 2013; Field et al., 2012, 2015a), suggesting a possible rise in C supply to AMF symbionts. However, this study found no increase in C flow to the wheat's mycorrhizal fungal partner at elevated [CO<sub>2</sub>], traced by directly measuring <sup>14</sup>C flow through the system over a 16-hour labelling (Figure 4.10). First, the <sup>14</sup>C is fixed by the wheat plants through photosynthesis, and then the photosynthate is transferred below ground to the plant roots and the plant-fungal partners. Earlier experiments using <sup>14</sup>C labelling showed that plant-fixed C could be transferred from the host plants to the mycorrhizal partner within hours (Johnson et al., 2002), but this is species-specific. In other experiments (Field et al., 2012, 2015b), the transfer time can be variable dependent on vasculature and presence of stomata in the species being studied.

The lack of an increase in C allocation to the mycorrhizal partners is surprising as the plants grown in elevated [CO<sub>2</sub>] conditions contained a significantly larger amount of plant fixed C in both their shoot and root tissue (Figure 4.10). Plants had more C available at elevated [CO<sub>2</sub>], but the percentage of fixed C allocated to the extra-radical fungi within the soil didn't increase. In fact, in cv. Avalon allocation of plant C to the fungal partner decreases under elevated [CO<sub>2</sub>], despite the prediction that plants will have increased nutrient demands, so should invest more in mycorrhizal associations. However, some of the additional C assimilated in the roots of the wheat plants may be contained within fungal structures, as the C acquired by fungi within the root system cannot be distinguished and thus quantified via this method. If no more C was allocated to the fungal partner at elevated [CO<sub>2</sub>] within this experiment, it might explain why mycorrhizal colonisation did not increase in this system under elevated [CO<sub>2</sub>], in contrast to meta-analyses trends (Treseder, 2004; Alberton et al., 2005). However, the significantly reduced C allocation in cv. Avalon still didn't have a substantial influence of percentage root colonisation or extra-radical hyphae, suggesting the AMF were not limited by plant photosynthate under ambient [CO<sub>2</sub>] conditions (Gavito et al., 2002), or that carbon allocation to the fungal partner is not consistent and the allocation measured at the time of this study is not representative of allocation partners over the plant's lifetime.

Results from a previous study with the same experimental set-up found a >1000% increase in C allocation to fungal networks in the soil cores at elevated [CO<sub>2</sub>] (1500 ppm) compared to the ambient [CO<sub>2</sub>] treatment (440 ppm); in one liverwort species (*Preissia quadrata*) and two vascular plant species (*Osmunda regalis*, *Plantago lanceolata*) (Field et al., 2012). However, these studies focus on wild plant species and may differ from crop plants such as wheat which have a much lower mycorrhizal dependency (Tawaraya, 2003) (MD, the degree of plant growth change associated with arbuscular mycorrhizal fungi). My results are supported by recent studies on wheat which also demonstrate no increase in C allocation to fungal partners at elevated [CO<sub>2</sub>] (Thirkell et al., 2019).

Plants with extensive root systems such as wheat may not rely as much on mycorrhizal associations due to their higher ability to acquire nitrate and phosphate from the soil. Alternatively, modern breeding techniques which have seldom focused on maintaining or improving crop associations with mycorrhizal symbionts, and in some cases centred on resistance to fungal pathogens, may have been detrimental to the function of the symbiosis (Toth et al., 1990). Modern wheat breeding has also focused on varieties which maximise investment in shoot biomass to increase yields, and therefore C investment below-ground is not prioritised within these varieties (Waines & Ehdaie, 2007).

The wheat plants tested here allocated only a small percentage (<1%) of their recently fixed C to extraradical AMF mycelium, at the time point measured. Therefore, the growth depressions observed when plants made associations with AMF are unlikely to be due to a large C drain on the plant. C allocation from the plant to the mycorrhizal fungi has previously been estimated at between 4-20% (Smith & Read, 2008); and a recent <sup>13</sup>C tracing experiment observed 4.3% of plant-fixed C being allocated to the fungal hyphae over 24 hours (Tomè et al., 2015). However, modern wheat cultivars have been shown to move minimal amounts of C below ground, and therefore this might account for the differences between the values in this study and previous studies (Keith et al., 1986; Gregory & Atwell, 1991; Thirkell et al., 2019). Additionally, studies of modern wheat cultivars have found that as wheat progresses through the growth stages it substantially reduces C investment below ground, so that by early booting stages 90% of C is retained in the shoot material and by flowering <5% is allocated below ground (Keith et al., 1986; Gregory & Atwell, 1991). Therefore, the minimal

investment of C to the AMF in this study may have been higher in the early plant growth stages, emphasising the importance of periodic measurements through the life-cycle.

The growth depressions caused by mycorrhizal fungi at ambient [CO<sub>2</sub>] were slightly mitigated at elevated [CO<sub>2</sub>], suggesting more C available within the plants may have some positive effect. Other studies in Citrus and *B. distachyon* have also shown elevated [CO<sub>2</sub>] can alleviate growth depressions caused by AMF at ambient [CO<sub>2</sub>] (Jifon et al., 2002; Jakobsen et al., 2016).

#### **4.5.2.3 The effect of [CO<sub>2</sub>] on plant phosphorus and fungal acquired <sup>33</sup>P.**

In cv. Skyfall, <sup>33</sup>P transferred from fungus to plant accounted for around 8.4% of the total <sup>33</sup>P added to the plant pots. However, there were substantial cultivar differences in mycorrhizal acquired P, with cv. Avalon only acquiring an average of 1.7% of the total <sup>33</sup>P added to the plant pots. Compared to the results in Chapter 2, cv. Skyfall had 2.5 times more AM-acquired P, suggesting the low nutrient substrate used in this experiment may have increased cv. Skyfall reliance on AMF. However, cv. Avalon had 1.5 times lower AM-acquired P in this experiment. As suggested in Chapter 3, cultivar differences could be due to cultivar-specific responses to the *R. irregularis* inoculum used in this experiment.

Despite mycorrhizal contribution to P uptake, particularly in cv. Skyfall, total P within the shoot material, in terms of absolute quantities or when normalised to plant biomass, was decreased in the presence of mycorrhizal fungal symbionts when compared to non-mycorrhizal control plants. This reinforces evidence that suggests a lack of growth or P uptake in mycorrhizal plants does not inevitably mean that mycorrhizal fungi have not contributed to P-uptake (Smith & Smith, 2011). However, in this case, it does suggest downregulation of the direct plant uptake pathway, which is not compensated for by the symbiosis at the time point measured (Smith et al., 2011).

The low amounts of mycorrhizal-acquired <sup>33</sup>P suggest the symbiosis between cv. Avalon and *R. irregularis* may not be nutritionally mutualistic at the time point measured, at either ambient or elevated [CO<sub>2</sub>]. Avalon has a much larger allocation of its biomass to the root system than cv. Skyfall, therefore, may rely less on mycorrhizal associations for nutrient uptake. At elevated [CO<sub>2</sub>] cv. Avalon may invest less in its fungal partner and more in its root nutrient uptake, although

the reduction in C allocation to the fungal partner at elevated [CO<sub>2</sub>] does not affect mycorrhizal acquired P or colonisation, suggesting the AMF may have been in a C surplus (Gavito et al., 2002), or received more C at other time points not measured in this experiment.

Elevated [CO<sub>2</sub>] did not affect <sup>33</sup>P transfer to the plants via mycorrhizas. Other studies which manipulated wheat C availability for mycorrhizas through shading showed similar results (Stonor et al., 2014). Shading reduced plant C availability but did not result in reduced colonisation by the mycorrhizal fungi or any change of plant growth responses to AMF, which remained negligible. *R. irregularis* continued to supply up to 28% of shoot P with no significant effect of reduced C availability through shading. However, it is essential to note that shading experiments have an inevitable impact on plant physiology, including reduced respiration and transpiration rates to compensate for lower rates of photosynthesis, which may partially negate reduced C availability.

Overall, total P within the shoot plant tissue increased at elevated [CO<sub>2</sub>], but this increase in P uptake could not be attributed to the AMF partner, at the time point measured. Similarly, elevated [CO<sub>2</sub>] did not affect the contribution of the AMF pathway to P uptake in *M. truncatula*, *B. distachyon*, *Pisum sativum* (Gavito et al., 2002; Jakobsen et al., 2016). However, results can vary between plant groups, in non-vascular liverworts, high [CO<sub>2</sub>] increased P gain by the plant shoots, but again in vascular plants such as ferns, angiosperms and wheat, there were no increases in fungal acquired P to the plants at elevated [CO<sub>2</sub>] (Field et al., 2012; Thirkell et al., 2019).

Additionally, the point in the plant lifecycle at which an experiment is conducted may change the effect of [CO<sub>2</sub>] on mycorrhizal P uptake. For example, in *P. sativum* (Gavito et al., 2002), *P. lanceolata* and *T. repens* (Staddon et al., 1999), P uptake in mycorrhizal plants decreased at the later time points measured under elevated [CO<sub>2</sub>]. Therefore, as mentioned earlier it is essential to monitor P assimilation by AMF across the life cycle of plants.

#### **4.5.2.4 The effect of [CO<sub>2</sub>] on plant nitrogen and fungal acquired <sup>15</sup>N.**

Total plant tissue N generally increased with elevated [CO<sub>2</sub>], whereas the concentration of N decreased. cv. Avalon grew >40% larger at elevated [CO<sub>2</sub>] despite only a 4% increase in absolute N concentrations, suggesting mycorrhizal

cv. Avalon had a rise in nutrient use efficiency (NUE) at elevated [CO<sub>2</sub>]. This is supported by previous research which showed wheat in association with *R. irregularis* had much higher NUE at elevated [CO<sub>2</sub>] than non-mycorrhizal wheat, even though no differences were found at ambient [CO<sub>2</sub>] resulting in a significant interactive effect between AMF and [CO<sub>2</sub>] treatments (Zhu et al., 2016).

This study confirmed the results in Chapter 2 that *R. irregularis* may contribute to plant N nutrition through uptake and transfer of ammonium to the plant partner. Previous studies have shown AMF ability to transfer both inorganic and organic N to a plant partner (Jin et al., 2005; Tanaka & Yano, 2005; Thirkell et al., 2016). However, there was no effect of higher [CO<sub>2</sub>] on mycorrhizal acquired <sup>15</sup>N, at the time point measured. This is corroborated by another study on wheat which found that although AMF could have a considerable positive impact on plant biomass responses to elevated [CO<sub>2</sub>], there was no significant increase in N uptake, either when measured as total plant N or hyphal uptake of <sup>15</sup>N (Zhu et al., 2016).

cv. Avalon seemed to gain less absolute <sup>15</sup>N through its mycorrhizal partner than cv. Skyfall at elevated [CO<sub>2</sub>], further suggesting cultivar-specific differences in the functionality of the symbiosis between *R. irregularis* and modern wheat. Results from Chapter 2 support these results, as inoculated cv. Skyfall acquired over twice as much AM acquired <sup>15</sup>N than inoculated cv. Avalon. However, there were also differences between the two experiments, with inoculated cv. Skyfall gaining more than twice as much <sup>15</sup>N through an AM partner in Chapter 2, and inoculated cv. Avalon gaining over three times as much AM acquired <sup>15</sup>N in Chapter 2. AMF are known to have high N demands (Johnson, 2010), therefore the low nutrient substrate used in this experiment may have reduced <sup>15</sup>N supply from the AMF to their plant host.



## 4.6 Conclusions

The objective of this study was to quantify the ability of a commercially-available AMF inoculum (*R. irregularis*) to contribute to improving wheat growth responses to elevated [CO<sub>2</sub>], either through enhanced nutrient acquisition or reduced cost of the symbiosis. I found that AMF could influence plant growth responses to [CO<sub>2</sub>], but it was wheat cultivar dependent. In cv. Skyfall and Avalon growth response to elevated [CO<sub>2</sub>] was more substantial when the plants were mycorrhizal. Whether [CO<sub>2</sub>] and AMF interact within experiments is highly variable, with some studies concluding that the function of AMF and its effects on plants are not affected by rising [CO<sub>2</sub>] (Jongen et al., 1996; Staddon et al., 1999; Gavito et al., 2000), and others supporting an interaction (Gavito et al., 2002; Hartwig et al., 2002; Zhu et al., 2016).

Two common hypotheses are that an interaction between AMF and [CO<sub>2</sub>] should occur to increase the 'C fertilisation effect' on plant growth; due to either an alleviation of nutrient limitations with AMF associations (Cavagnaro et al., 2011), or a re-balancing of source-sink dynamics with AMF increasing their sink strength at elevated [CO<sub>2</sub>] and preventing acclimation of photosynthesis at elevated [CO<sub>2</sub>] (Gavito et al., 2019). The results of this study support neither of these hypotheses.

Firstly, there was no evidence supporting an increase in AMF C sink strength at elevated [CO<sub>2</sub>], neither mycorrhizal biomass nor C allocation from the plant to the fungi increased at elevated [CO<sub>2</sub>]. Therefore, although plant C fixation increased at elevated [CO<sub>2</sub>], this did not lead to enhanced C allocation to fungal partners or an increase in fungal biomass. Therefore, hypotheses 1 and 2 which stated that wheat would have improved C fixation at elevated [CO<sub>2</sub>], and this will drive higher C allocation to fungal symbionts resulting in more abundant fungal biomass, are not supported.

Secondly, there was no observed increase in mycorrhizal acquired P or N at elevated [CO<sub>2</sub>]; therefore, AMF are unlikely to have offset any additional nutrient requirements at elevated [CO<sub>2</sub>]. Consequently, this study also found no support for hypothesis 3, that [CO<sub>2</sub>] would result in increased plant nutrient acquisition through the fungal partner.

Contrary to hypothesis 4, which predicted plant responses to AMF and atmospheric [CO<sub>2</sub>] would change over time due to plant acclimation to elevated

[CO<sub>2</sub>] or enhanced plant nutrient demand as they enter the reproductive growth stage; responses remained similar between the eight and ten weeks timepoints. There was no evidence that plants were acclimating to elevated [CO<sub>2</sub>] as the difference in biomass between plants grown at ambient and elevated [CO<sub>2</sub>] increased between eight and ten weeks. Equally, AMF did not seem to become more beneficial to the plant cultivars over time.

However, an enhanced 'C fertilisation' was, to some extent, achieved under nutrient-limited conditions due to AMF associations, in cv. Avalon and Skyfall. Hypothesis 5 predicted cultivar differences may stem from differences in AMF-acquired nutrients between the cultivars. However, cv. Avalon had a more substantial increase in growth at elevated [CO<sub>2</sub>] despite cv. Skyfall gaining significantly more AMF acquired P and N at elevated [CO<sub>2</sub>], therefore this hypothesis is not supported.

The enhanced 'C fertilisation' at elevated [CO<sub>2</sub>] may have been due to AMF increasing plant nutrient use efficiency under elevated [CO<sub>2</sub>]. If AMF can enhance growth responses to rising [CO<sub>2</sub>] but not enhance plant nutrient uptake, they may further decrease the nutritional value and lower the protein content of cereal crops, which is undesirable from a food security perspective (Myers et al., 2014).

This study observed growth depression in mycorrhizal wheat compared to non-mycorrhizal wheat, and a cultivar-dependent lessening in these growth depressions at elevated [CO<sub>2</sub>]. A reduction in biomass losses due to AMF under high [CO<sub>2</sub>] conditions has been observed in previous experiments (Jifon et al., 2002; Jakobsen et al., 2016), and is often predicted to be associated with a reduced cost of the symbiosis at elevated [CO<sub>2</sub>] due to surplus C supplies. C fixation to the shoot and root material did substantially increase at elevated [CO<sub>2</sub>], with no coinciding increase in C allocated to the fungal partner. Therefore, C drain on the plant may have been reduced at elevated [CO<sub>2</sub>]. However, within these symbioses C allocated to the fungi was such a small percentage of the plant's C budget at the time point measured, it seems unlikely that growth depression was the result of a C drain on the plants. Growth depressions associated with AMF are more likely the result of a reduction in P uptake in the mycorrhizal plants, due to the suppression of the direct uptake pathway.

Considerable cultivar differences were observed in this study, supporting hypothesis 5. Cultivars responded differently to AMF and [CO<sub>2</sub>] interactions.

Additionally, carbon-for-nutrient exchange within the symbiosis was altered by rising [CO<sub>2</sub>] in a cultivar specific manner. We conclude the effect of [CO<sub>2</sub>] on AMF function will likely vary between plant and fungal taxa, and it would be unwise to generalise findings from specific plant-fungal species interactions. Therefore, studies should be conducted on a wide-range of plant-fungal symbioses under varying environmental conditions.

## **Chapter 5 : General Discussion**

A major risk to future food security is the decline of phosphate-rock resources used for the production of fertilisers and the parallel rising demand for these fertilisers to increase food production (Cordell et al., 2009; Godfray et al., 2010). The remaining reserves of phosphate-rock are concentrated in Morocco, China and the USA, which has the potential to cause political instability in those countries solely reliant on imports (Cordell et al., 2009). Additionally, the use of chemical fertilisers is becoming problematic due to the substantial amounts of N and P leached into the natural environment polluting vital water supplies (Robertson & Vitousek, 2009). A large proportion of the GHGs created during food production are a result of manufacturing N fertilisers using the Haber-Bosch process (Tubiello et al., 2013). It is critical GHG emissions in agriculture are substantially reduced if targets of net-zero CO<sub>2</sub> emissions by 2050 are to be achieved and the disastrous consequences of climate change avoided (IPCC, 2018).

The majority of essential crop plants across the globe form symbioses with AMF (Smith & Read, 2008), and it has recently been shown that, for wheat, AMF can transfer P and N to their host plant in exchange for photosynthetically-fixed plant C (Thirkell et al., 2019). As such, there is an excellent potential for exploitation of AMF in agriculture to reduce chemical fertiliser usage. Crop nutrient uptake through AMF associations could be enhanced by breeding future crop cultivars to be more receptive to AMF associations. My research has shown that currently, wheat cultivars have low C investment in the symbiosis, and receive relatively low benefits from associations in terms of nutrient assimilation, although variations between wheat cultivars means there is potential to breed for cultivars which gain more from AMF associations. Current breeding programmes have never considered traits associated with mycorrhizal symbioses. They may have even inadvertently reduced compatibility and function of crop-AMF associations by selecting for resistance to fungal pathogens and focusing on yield-related traits, thereby cutting plant investment in below ground structures. Breeding could be combined with the addition of AMF inoculum to agricultural soil to reduce the risk of P limitation in crops and reduce the need for P-based chemical fertiliser application.

Over the last decade, growing numbers of commercial companies are producing and selling AMF inoculants to meet increasing demand from farmers (Vosátka et al., 2012; Faye et al., 2013). However, plant benefit from the symbiosis can vary, often according to species or cultivar (Klironomos, 2003; Hoeksema et al., 2010); bringing into question the advantage of widespread inoculant applications. There is still little understanding about what causes the variety of responses AMF elicit from their host plants, and this is a critical knowledge gap preventing the broader application of AMF technologies. Moreover, the responsiveness of cereal crops, such as wheat to AMF, has been highly debated (Ryan & Graham, 2018; Rillig et al., 2019). Many studies suggest AMF will be unimportant in cereal agriculture as they are generally negatively or neutrally affected by AMF colonisation (Ryan & Graham, 2018). However, a recent study has shown that wheat yields and nutrient uptake could be considerably increased after AMF inoculation (Zhang et al., 2019). Indeed, a recent investigation of AMF contribution to P and N acquisition in wheat demonstrated that although AMF contributed to plant nutrient assimilation, it varied according to cultivar (Thirkell et al., 2019). Therefore, there is an urgent need to build on this study by assessing how commercially-available AMF inoculum could function across wheat cultivars to determine whether AMF inoculation should be implemented in future agricultural systems. My research aimed to address this knowledge gap by assessing the efficacy of commercially-available mycorrhizal inoculum in terms of receptivity, costs and benefits derived from the association in three cultivars of wheat (Chapter 2).

Native AMF communities are already present in agricultural soils, although they may be depleted due to destructive farming practices such as tillage, fungicide use, and long fallow periods (Helgason et al., 1998; Daniell et al., 2001). Therefore, it is important understand how “foreign” AMF genotypes added through commercial inoculation will interact with native AMF species (Rodriguez & Sanders, 2014) and, how the resultant AMF community hosted within the plant root will change. If inoculation results in AMF-acquired benefits to the plant host, it is vital to understand whether this is a direct effect from the introduced AMF or whether indirect changes in the local AMF or broader rhizosphere microbial community could be responsible for the benefits (Rodriguez & Sanders, 2014). My research has explored this issue using T-RFLP analyses of the fungal communities colonising the roots of three cultivars of wheat in agricultural soil with and without the addition of commercially-available AMF inoculum.

Compounding the problems threatening global food security, climate change is now also negatively impacting agricultural productivity (Miyan, 2015; Zhao et al., 2017b), and we know little about how increasing [CO<sub>2</sub>] will change the functionality of mycorrhiza-crop relationships. [CO<sub>2</sub>] has previously been shown to be a potent regulator of mycorrhizal function in non-crop plants (Field et al., 2012). Therefore, as the IPCC projects continued atmospheric [CO<sub>2</sub>] increases, we must understand how the symbiosis between crops and AMF will respond to this future climate. By investigating the receptivity and functioning of mycorrhizal fungi in wheat with and without the addition of commercial inoculum, my research has sought to address this major gap by assessing the impact of rising atmospheric [CO<sub>2</sub>] on the symbiosis.

My research has sought to determine the effectiveness of commercially available AMF inoculants in facilitating mycorrhizal nutrient assimilation in wheat, an essential UK crop plant, thereby reducing the need for fertiliser applications at current rates. Additionally, by measuring the functionality of wheat-AMF associations at multiple atmospheric CO<sub>2</sub> concentrations, I investigated how wheat-AMF symbiosis will respond to future climate change, thereby assessing whether AMF could play a role in alleviating the impacts of climate change on UK agriculture. The results of my research could translate into part of a sustainable agriculture management strategy and provide a vital assessment of the growing AMF inoculant industry.

Using simultaneous isotope tracers (<sup>14</sup>C, <sup>33</sup>P and <sup>15</sup>N), I measured C-for-nutrient exchange between wheat and their AMF symbionts following the addition of a commercially available mycorrhizal inoculum (*R. irregularis*), or a sterilised carrier substrate as control, to a non-sterile agricultural soil. I quantified whether the response to *R. irregularis* inoculum was cultivar-specific using the three, elite wheat cultivars Avalon, Cadenza and Skyfall. cv. Skyfall was selected as it is the UK's most widely grown wheat cultivar. cv. Avalon and Cadenza are parent lines in current breeding programs with high density maps of genetic markers, they were also originally chosen due to the large phenotypic differences between the cultivars. Preliminary data also demonstrated variation in AMF receptivity (unpublished). Using T-RFLP community composition analysis, my results suggest that the AMF species introduced via the addition of active inoculum established in the soil and successfully colonised the roots of the wheat cultivars

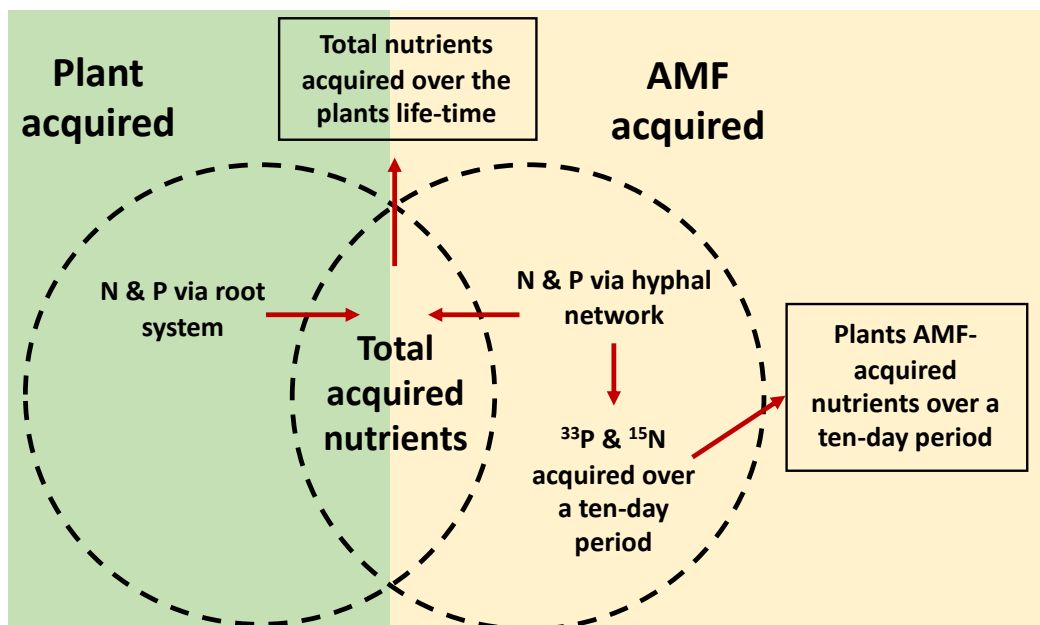
tested. Therefore, there is an increased likelihood that any responses of the wheat cultivars to inoculation were directly due to *R. irregularis* colonisation rather than any facilitation effects on the native AMF community. Finally, I quantified changes in C-for-nutrient exchange between the cultivars, with and without active inoculation, at elevated [CO<sub>2</sub>] (in line with IPCC projections for future [CO<sub>2</sub>]).

## **5.1 Commercial AMF inoculum increases root colonisation but not the assimilation of mycorrhizal-acquired nutrients.**

The majority of research which shapes our understanding of the potential of AMF inoculum to improve plant growth and nutrient uptake compares inoculated plants to non-mycorrhizal control plants (Lekberg & Koide, 2014b). However, in reality, cereal crops such as wheat are highly unlikely to remain uncolonised by AMF during field cultivation, where communities of AMF are present in the soil (Helgason et al., 1998). Therefore, to assess the realistic benefits of AMF inoculum application in agriculture, it is essential to investigate the benefits (or costs) of inoculation using non-sterile soil from an agricultural field where a native AMF community is present. I used this rationale in my experiments to investigate the efficacy of commercial AMF inoculants in agricultural systems and provide an accurate assessment of any enhancements in growth and nutrient uptake, which could be derived from inoculum use.

In Chapters 2 and 3 I showed that generalist AMF species such as *R. irregularis* could successfully colonise multiple wheat cultivars (see Chapter 2 and 3) in non-sterile systems where a native AMF community was present in the soil. This finding alleviates fears somewhat that inoculum may lose viability during production and storage, making it unfeasible for use in agriculture (Vosátka et al., 2012). The successful establishment and colonisation of wheat cultivars by *R. irregularis* from inoculation also provides evidence that AMF grown in controlled cultures for many generations can be compatible with a new target environment (Rodriguez & Sanders, 2014). Therefore, generalist species such as *R. irregularis* may be adaptable to other environmental conditions they have not previously experienced while in culture. However, my research brings up some concerns about contamination within the inoculum production process, which is often not sterile, as there was a T-RF in the inoculum which could not be associated with *R. irregularis* (see Chapter 3). High-quality control is essential, as for the inoculum industry to be successful consumer trust in the product is critical. Previous issues

have been identified with companies selling products not containing the AMF species they advertise (Faye et al., 2013). Contamination and low-quality control within inoculum products could result in a financial penalty to growers, as these products are expensive and may produce no increases in colonisation or AMF associated benefits. Alternatively, there is even a risk of yield penalties if inoculants are contaminated with pathogenic organisms. However, the results in Chapter 2 showed that increased colonisation by AMF does not relate to increased function and the amount of AMF acquired P and N supplied to the plant partner.



**Figure 5.1: The plant's total acquired nutrients vs the plant's mycorrhizal acquired nutrients measured through isotope tracing.** Total nutrient content within the plant tissues is the culmination of plant-acquired nutrients and AMF-acquired nutrients over the entire growth period. In contrast, AMF-acquired nutrients are measured via <sup>33</sup>P and <sup>15</sup>N accumulation in the plant tissue over a limited ten day period before plant harvest.

The addition of active commercial *R. irregularis* inoculum to agricultural soil resulted in a substantial increase in total plant P uptake, from combined plant and fungal uptake pathways (Figure 5.1) within the wheat cultivars tested. Enhanced P uptake in inoculated plants, could have important implications for reducing the use of phosphorus-based fertiliser in agriculture, which is essential for a sustainable farming system in the future. Vital next steps in this research are to test whether this increase in total P uptake with inoculation can be achieved in



the field, as has been shown in other studies (Mohammad et al., 2004). However, despite higher [P] within the inoculated wheat tissue, we found no corresponding increase in  $^{33}\text{P}$  uptake and transfer to inoculated plants via the AMF hyphae (Figure 5.1), leaving the mechanism behind the rise in plant tissue P unknown. Previous studies have also reported significant increases in plant P uptake with AMF inoculation, despite finding low P uptake through the mycorrhizal pathway (Pearson & Jakobsen, 1993a; Smith et al., 2003). These results suggest an increase in the plant's direct uptake pathway may occur, but the mechanism requires further research.

One possible explanation for the increase in direct P uptake upon inoculation could be a change in the microbiome within the bulk soil or the rhizosphere associated with AMF. Previous research has shown PSB have the potential to improve P availability to the plant via solubilising unavailable forms of P (Chen et al., 2006), and PSB have been detected in intimate association with AMF hyphae (Zhang et al., 2014). PSB may use C exudates from AMF hyphae, and therefore a substantial increase in AMF density in the soil due to inoculation may enhance C exudates and solubilisation of P (Zhang et al., 2018). Alternatively, increased root colonisation by AMF can enhance stomatal conductance (Augé et al., 2014) which could have knock-on effects on transpiration and mass-flow of nutrients towards the plant roots (Hepworth et al., 2015). Changes in stomal conductance and AMF colonisation can even result in changes to root architecture (Wu et al., 2013; Hepworth et al., 2016). All of the above could enhance nutrient uptake through the direct-uptake pathway, but confirmation through further studies is essential.

Future research should focus on how the addition of AMF inoculum affects the microbiome in the bulk soil and the rhizosphere. For example, research should quantify if there are any increases in PSB, which could be responsible for increases in plant P uptake (Zhang et al., 2018). It will also be essential to study whether AMF community changes induced through inoculation will result in other microbiome community changes which could have knock-on impacts on the plant host (Kobae, 2019).

Although labour intensive and costly, it would be beneficial to measure changes in the function of the symbiosis across time to quantify whether the contribution of the AMF pathway to plant P uptake was greater at an earlier point in the plant's

lifecycle. For example, AMF associations could be most beneficial before the plant's root system, and photosynthetic material is fully established, as AMF hyphae are much smaller and therefore can proliferate faster through the soil, acquiring nutrients for the plant at a presumably much lower C cost (Chen et al., 2016). This is exemplified in orchids, which have tiny seeds with few reserves to support seedling development, making them reliant on mycorrhizal fungi for nutrients and C in early life stages. Orchids only become fully-autotrophic after the appearance of green above-ground material, at which time they are less dependent on the fungal partner and C flow may begin from the plant to the fungus (Cameron et al., 2006). When fully developed the plant root system may be sufficient to obtain the plant's P requirements, leading to a suppression of the mycorrhizal uptake pathway through reduced C investment in the symbiosis. This may be especially true in cereal crops which have been bred to have an extensive root system of fine roots, optimised for nutrient acquisition in environments where nutrients are readily available due to fertiliser use (Smith & Smith, 2011; Mai et al., 2018; Zhang et al., 2019).

Consistent with the results of my research (see Chapter 2), there are examples in the literature where AMF have a neutral effect on plant biomass despite enhancing plant P uptake (Wilson & Hartnett, 1998; Tawaraya, 2003; Klironomos, 2003), suggesting AMF inoculation may be an unreliable method to improve food security. Further research is required to clarify what determines plant biomass responses to AMF, beyond AMF species identity. For example, higher nutrient uptake in inoculated plants might be counteracted by an increased C drain on the plant and offset positive biomass responses. However, there was no evidence of a rise in C allocation to the fungal partner after inoculation of wheat in agricultural soil in my experiments (see Chapter 2).

Furthermore, my experiments provided very little evidence that AMF associations act as a significant C drain on the plant in general. Often, the extra-radical AMF hyphae acquired less than 1% of the plants C assimilated during one photoperiod (see Chapters 2 and 4). Under the conditions of my experiment, it appears that N rather than P was the plant's limiting nutrient. In future agricultural environments, it is more likely that P would be the limiting nutrient as it is a non-renewable resource with production expected to peak by 2033 (Cordell et al., 2009). In a P limited scenario, AMF inoculation would more likely have resulted

in plant biomass increases. Inoculation with *R. irregularis* did not have any effect on plant N uptake; potentially explaining the lack of plant biomass increases. Therefore, my experimental evidence adds to the growing case that AMF may be more critical for P uptake than N uptake. P is relatively immobile in the soil, and consequently, as plants take up P in their immediate surroundings, they create a depletion zone around their roots (Figure 1.1) (Smith & Read, 2008). AM hyphae can extend past a plants depletion zone giving access to previously unavailable P. However, inorganic forms of N are relatively mobile in the soil and transported to the roots by mass flow, so depletion zones are not considered a significant issue unless the land is dry (Smith & Read, 2008).

Addition of commercial inoculum to the soil likely caused a substantial boost in spore density, increasing the soil inoculum potential (Lekberg & Koide, 2005). Given that AMF rely solely on host plant C resources, C supply below ground may limit colonisation of roots. However, my research provides evidence that this is often not the case. I found a substantial increase in AMF colonisation of roots, with no increase in C flow to the root system or extra-radical hyphae (see Chapter 2). Higher C investment in the AMF partner may have occurred at an earlier stage in the plant's life cycle, and therefore would have been missed at the eight week time point measured in this experiment. However, extra-radical hyphal network length did not increase significantly within any wheat cultivar. Extra-radical hyphal networks are responsible for foraging in the soil for nutrients to pass to the plant host and are in turn supported by the host plant, therefore could be indicative of plant C investment in the longer term.

## **5.2 Commercial AMF inoculum alters the community colonising roots, with little variation between wheat cultivars.**

The addition of commercial AMF inoculum to agricultural soil can result in changes to carbon-for-nutrient exchange within the wheat-AMF symbiosis (see Chapter 2). To interpret whether these functional changes to the symbiosis are a direct result of the inoculated species or an indirect result of changes to the wider native AMF community, I tracked the establishment of the inoculum and any resulting changes to the AMF community in the soil (Chapter 3).

Higher root colonisation within inoculated plants is not a good indicator of inoculum establishment. For example, even when the level of colonisation was

unchanged in inoculated plants, the community within the roots was dramatically different (see Chapter 3). Therefore, differences in root colonisation between inoculated and non-inoculated plants are not likely to be due to differences in the ability of *R. irregularis* to colonise the various wheat cultivars. The colonisation was between 54 -178% higher in inoculated plants and differed widely between the wheat cultivars (see Chapter 2). When the inoculum density in the soil was no longer limiting colonisation, C flow below ground could have had a more significant impact. Avalon had the highest C allocation to the AMF, regardless of inoculation treatment, and also, the highest increase in root colonisation after inoculation (see Chapter 2). Previous studies have shown a tremendous difference in wheat cultivar colonisation with AMF (Zhu et al., 2001; Lehnert et al., 2017). Such variation may in part be due to genotypic differences in the ability of wheat to form AMF symbiosis, given that 30 genetic markers have been associated with the extent of root colonisation in wheat by AMF (Lehnert et al., 2017).

Both facilitation and competition interactions can occur between the foreign AMF species applied through commercial inoculum and native species (Callaway & Walker, 1997; Thonar et al., 2014). Therefore, it is important not to presume that any change to the AMF community colonising the root will be driven exclusively by the species within the inoculum. My results show that upon inoculation with *R. irregularis*, the AMF community colonising the roots became dominated by *R. irregularis* (see Chapter 3). However, another T-RF likely representing a species from the native AMF community, also had an increased presence in inoculated plants, providing evidence that facilitation or coexistence may occur between some species. This trend was seen across all wheat cultivars, as there was no significant difference in the AMF communities after inoculation. It is unknown why some species of AMF compete more strongly, while others can coexist. One hypothesis is that species with different niches will avoid competition and better coexist (Vandermeer, 1972), and this has been tested through measuring communities phylogenetic relatedness.

In some cases, communities with more distantly related species maintained higher species richness (Maherali & Klironomos, 2007), suggesting a higher ability to coexist. As AMF traits are phylogenetically conserved (Powell et al., 2009), more distantly related species with different characteristics may

experience reduced competition. However, other studies show AMF communities are more phylogenetically clustered than would be expected by chance (Horn et al., 2014). Many processes could produce clustering, including a powerful environmental filter favouring species with similar traits (Maherali & Klironomos, 2012), or biotic factors such as interactions with the host plant or the wider soil biotic community selecting for phylogenetically related symbionts (Horn et al., 2017). More in-depth experiments into how the AMF community changes after inoculation using next-generation sequencing (NGS) technologies, could determine if inoculation results in facilitation of species which are close relatives or phylogenetically diverse.

A large proportion of the variation between the communities of AMF within the plant roots was due to inoculation treatment (56%, PERMANOVA). The addition of a commercial inoculum containing *R. irregularis* resulted in the displacement of the dominant native species (*Funneliformis* species) with *R. irregularis*. This shift in the dominant species colonising the plant roots could have significant functional implications for the plant host. Different AMF species/isolates can have vastly different effects on their plant host due to both differences in their compatibility and their functional capacities (Klironomos, 2003; Mensah et al., 2015). For example, a study measuring the transcriptional changes in cassava colonised with two genotypes of *R. irregularis* found considerable variation in the transcription of genes associated with the symbiosis. This suggests the plants had different responses to the two isolates and cassava cultivars responded differently; however, the consequences of this on symbiotic function were not measured (Mateus et al., 2019). Other studies have shown significant differences in the functional capacities of different AMF isolates to increase N uptake in *Medicago sativa*, from no impact on plant tissue N to substantial increases of over 200% (Mensah et al., 2015).

My research has demonstrated functional changes to the symbiosis that could be due to a cultivar-specific response to inoculation with *R. irregularis* (see Chapter 2). One wheat cultivar (cv. Avalon) showed a significant decrease in the acquisition of <sup>15</sup>N and <sup>33</sup>P isotopes through a fungal partner after inoculation with *R. irregularis*. This decline in symbiotic functioning is not present in the other wheat cultivars tested, which maintained a similar carbon-for-nutrient exchange despite incurring drastic changes to the AMF community colonising their roots.

Cultivar specific responses to *R. irregularis* and other AMF isolates have been shown previously (Singh et al., 2010; Chu et al., 2013), for example when the same AMF inoculant colonised five Maize cultivars, positive, neutral and negative responses were all observed (Chu et al., 2013).

The negative cultivar-specific response to *R. irregularis* is further supported by evidence in Chapter 4, which also demonstrated that cv. Avalon had a much lower acquisition of  $^{33}\text{P}$  than cv. Skyfall when colonised exclusively with *R. irregularis*. At elevated  $[\text{CO}_2]$ , cv. Avalon also had a significantly lower acquisition of  $^{15}\text{N}$ . Although *R. irregularis* is a widely studied generalist species and the most common species used in inoculation studies, this genotype may not be a favourable symbiont for all wheat cultivars. As genotype-specific differences have shown to be vast, other genotypes may be more favourable (Croll et al., 2008; Angelard et al., 2010). Research should continue to pursue small-scale tests on inoculant suitability to a range of plant hosts and environments before inoculants are applied at a farm scale.

The composition of the fungal communities detected within the wheat roots grown in nonsterile agricultural soil suggests that the soil contained a low diversity of AMF species (see Chapter 3). The low diversity of AMF species present indicates few species can survive the harsh environment of intensive agriculture (Helgason et al., 1998). Those that are present are likely to be highly tolerant of disturbance, less susceptible to high nutrient concentrations and have a high investment in reproduction (Jansa et al., 2002; Oehl et al., 2010; Verbruggen & Kiers, 2010).

The introduction of a high density of spores in the soil, close to the plant roots could have given the species within the inoculum a competitive advantage and consequently reduced the relative abundance of the dominant native AMF species (Hepper et al., 1988). However, longer-term field studies are required to monitor inoculum establishment and survival over multiple years. One field study which measured inoculum establishment over multiple years found that one out of the two introduced species persisted for at least two years following inoculation (Pellegrino et al., 2012). In the roots of none inoculated plants, within my study system, *R. irregularis* was only present in small proportions. Therefore *R. irregularis* may not be competitive in this environment over the long term when subjected to regular disturbance events and changing environmental conditions typical of agro-ecosystems.

Land management intensity influences AMF diversity and community structure, and tillage has the most drastic effect on AMF communities within agricultural systems (Helgason et al., 1998; Jansa et al., 2002; Oehl et al., 2010; Verbruggen & Kiers, 2010). Agricultural soils are often found to be rich in *Glomeraceae* species, including *Glomus mosseae* (now *Funneliformis mosseae*), *Glomus caledonium* (now *Funneliformis caledonium*) and *Glomus group A*, and depleted in species of *Acaulospora*, *Gigaspora* and *Scutellospora* (Helgason et al., 1998; Daniell et al., 2001; Jansa et al., 2002; Oehl et al., 2003; Rosendahl, 2008). The disturbance tolerance of *R. irregularis* is less clear (Alguacil et al., 2014; Wetzel et al., 2014), with a recent study showing that *F. mosseae* and *F. caledonium* dominated in conventionally tilled plots, whereas *R. irregularis* was found more frequently in the no-till plots (Wetzel et al., 2014).

If the application of *R. irregularis* inoculum is to form part of a sustainable management system, it would have to be combined with changes in management strategies, for example reducing tillage and using cover crops (Lekberg & Koide, 2005). Initial applications of AMF inoculants to restore community diversity and abundance may be costly; however, with the adoption of non-harmful management strategies, repeat applications may not be necessary. However, if management strategies remain the same, there is a high chance the initial community structure will re-establish.

Simultaneously, it is crucial to consider concerns that AMF species introduced to new environments will be able to persist and may spread. If introduced species are highly competitive and not beneficial, they could become invasive in natural systems or problematic in agricultural systems, although there is little evidence to date that this has occurred (Hart et al., 2018). However, soil biodiversity including AMF diversity has a crucial role in influencing plant communities and ecosystem processes, such as nutrient and C cycling (Bardgett & Van Der Putten, 2014; Wagg et al., 2014). Recent studies have shown soil biodiversity is in decline (Bardgett & Van Der Putten, 2014), and models have shown that changes in these soil communities can damage many ecosystem functions, including the ability of the soil to cycle and retain nutrients which is vital for nutrient acquisition by plants (Wagg et al., 2014). Moreover, plants may be adapted to their local soil communities, including native AMF species (Johnson et al., 2010). For example, native AMF associated with grasses had ~90% higher hyphal lengths, suggesting

they can sequester more C (Johnson et al., 2010). Native AMF were also better mutualists in P-limited sites and less parasitic in the nitrogen-limited sites when their effect on plant inflorescence biomass was measured (Johnson et al., 2010). Therefore, inoculum use in agriculture should proceed with caution and requires further study into the possibility of spread into natural environments. In this case, I introduced a species already present within the environment. Nevertheless, it is unknown whether the same isolate was present; therefore, new genetic material could still have been introduced into the AMF community, and this should be tracked in the future through NGS techniques (Rodriguez & Sanders, 2014).

### **5.3 Carbon-for-nutrient exchange between AMF and wheat is not enhanced by rising [CO<sub>2</sub>].**

C3 plants, including wheat, generally fix more C at elevated [CO<sub>2</sub>], due to reduced photorespiratory losses and therefore, an enhanced net photosynthetic rate (Wang et al., 2013). In two wheat cultivars, C assimilated in the shoot material over one photoperiod was substantially higher at elevated [CO<sub>2</sub>], showing the plants were fixing more C as expected, and may have more resources available to invest in AMF partners (see Chapter 4). Whether increased C assimilation within the plant material results in increased C flow below ground to a plant's AMF partner appears to be highly species-dependent, with most previous studies showing an increased C flow below ground (Drigo et al., 2010, 2013; Field et al., 2012, 2015a), or an increase in mycorrhizal biomass indicative of increased C investment in the fungal partner (Drigo et al., 2007). In liverworts and non-crop vascular plants (*Preissia quadrata*, *Osmunda regalis*, *Plantago lanceolata*), elevated [CO<sub>2</sub>] conditions resulted in a substantial increase in C allocation to the extra-radical mycelium (ERM) of the AMF partner (>1000%) (Field et al., 2012). However, a recent study on three wheat cultivars showed no increase in C investment in AMF symbionts at elevated [CO<sub>2</sub>] (Thirkell et al., 2019), in agreement with my results (Chapter 4). Fungal biomass did not increase at elevated [CO<sub>2</sub>], and these fungal structures are supported by plant C resources and therefore indicate that C investment to the AMF did not increase at elevated [CO<sub>2</sub>] throughout the experiment. Thus, for wheat, C allocation to a fungal partner was not limited by the plants' C resources at ambient [CO<sub>2</sub>], and may instead be controlled independently of the plants own C resources and not just be a proportion of C assimilated by the plant (Thirkell et al., 2019).



The species-specific response to elevated  $[\text{CO}_2]$  is likely to be a result of intensive breeding in crop plants, including the wheat cultivars used in this thesis. Modern cereal crops are bred to maximise C allocation above ground into harvestable biomass (Siddique et al., 1990). In intensive agricultural systems, inorganic nutrients are readily available through the application of fertilisers and negate the need for investment in large root systems. Consequently, breeding programmes could have unintentionally selected against higher investment in AMF partners. Crops plants such as wheat have a much lower mycorrhizal dependency compared to wild plant species, possibly due to extensive and fine root systems compared to other wild species (Tawaraya, 2003).

However, at elevated  $[\text{CO}_2]$  the wheat plants were larger and so acquired more nutrients from the soil (see Chapter 4). Therefore, to capitalise on plant growth responses to rising  $[\text{CO}_2]$  more chemical fertilisers may need to be applied. Nutrient limitations often limit plant growth responses at elevated  $[\text{CO}_2]$ , and there is evidence that if plants use all available nutrients during the vegetative growth period, grain yields and nutrition could be negatively affected (Cavagnaro et al., 2011; Pandey et al., 2015). N tissue concentrations are often found to be reduced at elevated  $[\text{CO}_2]$  (Kitao et al., 2005; Xu et al., 2013; Bloom et al., 2014). Reduced N content risks decreasing the nutritional value of cereal crops and jeopardies the nutrition-related health of millions of people (Myers et al., 2014). Moreover, recent evidence suggests that even increasing N fertiliser application may not be sufficient to maintain  $[\text{N}]$  at similar levels to those seen at ambient  $[\text{CO}_2]$  (Pleijel et al., 2019). However, inorganic nutrient applications would exacerbate already excessive fertiliser use and exhaust limited P supplies at a faster rate, therefore more sustainable alternatives to aid plant nutrient uptake such as AMF inoculants must be sought.

Symbiotic fungi may play a role in supplementing plant nutrient uptake at elevated  $[\text{CO}_2]$ . However, there is no evidence that P or N supplied by AMF partners is enhanced at elevated  $[\text{CO}_2]$  (see Chapter 4). Plant assimilation of fungal-acquired  $^{33}\text{P}$  was reduced at elevated  $[\text{CO}_2]$  in cv. Avalon, and although this trend was not statistically significant, it was consistent with a recently published study using the same wheat cultivar (Thirkell et al., 2019). Despite trends reported in the literature towards increased C flow below ground at elevated  $[\text{CO}_2]$ , most research agrees

that AMF-mediated P uptake is not enhanced by elevated [CO<sub>2</sub>] (Gavito et al., 2002, 2003; Jakobsen et al., 2016).

Although plants gained P from AMF at both ambient and elevated [CO<sub>2</sub>], there were substantial cultivar differences in the amounts of P obtained via AMF associations. In cv. Skyfall, AMF supplied P accounted for around 8.4% of the total <sup>33</sup>P added to the plant pots, indicating that AMF can facilitate P acquisition of the plant in this case. However, cv. Avalon only acquired an average of 1.7% of the total <sup>33</sup>P added to the plant pots. These cultivar differences were consistent with the results in Chapter 2 and with recently published studies on wheat and AMF, where cv. Skyfall was calculated to have acquired 570 times more of the <sup>33</sup>P than cv. Avalon (Thirkell et al., 2019). There was no correlation between the amounts of AMF acquired nutrient's gained by the wheat and the C transfer to AMF partners. Therefore, the cultivar differences in P acquisition from AMF associations may instead be due to differences in plant-fungal compatibility (Walder & van der Heijden, 2015).

cv. Avalon appears to form a less nutritionally mutualistic association with *R. irregularis* than cv. Skyfall; this has the potential to explain the more substantial growth suppression seen in this cultivar when compared to NM control plants (see Chapter 4). Although, shoot biomass of both varieties tested was lower with AMF associations, cv. Avalon experienced growth depressions which were twice as large as cv. Skyfall. Evidence presented here supports the hypothesis that growth depressions are due to a reduction in the plants' direct uptake pathway rather than an excessive C drain from the AMF associations (Smith & Smith, 2011). For example, despite mycorrhizal contribution to P uptake, particularly in cv. Skyfall, total P within the shoot material in terms of absolute quantities or when normalised to plant biomass was decreased in the presence of AMF symbionts when compared to non-mycorrhizal control plants (see Chapter 4).

#### **5.4 Variability of plant-AMF interactions.**

Within the three data chapters of this thesis, and within the wider literature, there is a theme of inconsistency in the outcome of plant-AMF interactions. In this thesis, the same three wheat cultivars and the same brand and species of commercial AMF inoculum (PlantWorks, UK) were used throughout. However, plant responses were not consistent. For example, within Chapter 2 inoculation

of a non-sterile agricultural soil resulted in a large increase in plant phosphorus uptake, whereas in Chapter 3, no increase in phosphorus uptake was achieved after inoculation. Both experiments had an increase in wheat root colonisation after inoculation, suggesting the inoculum was viable. However, the experiments were conducted at different times of the year, which could have affected the communities of native AMF or the density of AMF spores within the experiments (Daniell et al., 2001; Cotton et al., 2015). Soil collected in Experiment 2, in February, produced higher root colonisation than the soil collected in Experiment 3, in May, suggesting different AMF propagule abundance in the soil. If intra/interannual variation in AMF soil communities and propagule abundance can substantially alter host plant responses to inoculation, this could have implications for their use by farmers, as major concerns could be raised about their reliability.

The plants within Experiments 1 and 2 were grown in a semi-controlled greenhouse environment therefore, the climate and light conditions may have varied throughout the year. Numerous studies have shown that increases in soil temperature can result in higher root colonisation by AMF (Staddon et al., 2004). AMF may only start acquiring nutrients for a host plant once temperatures have reached a certain level. For example, at temperatures below 15°C P transfer to the plant was not enhanced over non-AM plants (Karasawa et al., 2012), possibly due to the complete suppression of ERM growth below 15°C in some species (Gavito et al., 2005). One study identified 18°C as an optimum temperature for plant biomass and P gain with higher and lower temperatures (11°C and 21°C) negatively effecting the relationship (Barrett et al., 2014).

Chapter 4 also used the same cultivars and inoculum but a different growth substrate, sand and perlite, which is likely lower in nutrients and also didn't contain a native AMF community. Compared to the results in Chapter 2, cv. Skyfall had 2.5 times more AM-acquired P, suggesting the low nutrient substrate used in this experiment may have increased cv. Skyfall reliance on AMF. However, cv. Avalon had 1.5 times lower AM-acquired P in this experiment. As suggested in Chapter 3, cultivar differences could be due to cultivar-specific responses to the *R. irregularis* inoculum used in this experiment. Therefore, cv. Avalon's AMF acquired P may have been reduced due to the lack of a native AMF community in Chapter 4.

However, contrary to AMF acquired P, inoculated cv. Skyfall gained more than twice as much N through an AM partner in Chapter 2 compared to Chapter 4. This pattern was also true for inoculated cv. Avalon which gained over three times as much AM acquired N in Chapter 2 compared to Chapter 3. AMF are known to have high N demands (Johnson, 2010), therefore the low nutrient substrate used in this experiment may have reduced N supply from the AMF to their plant host.

## 5.5 Conclusion

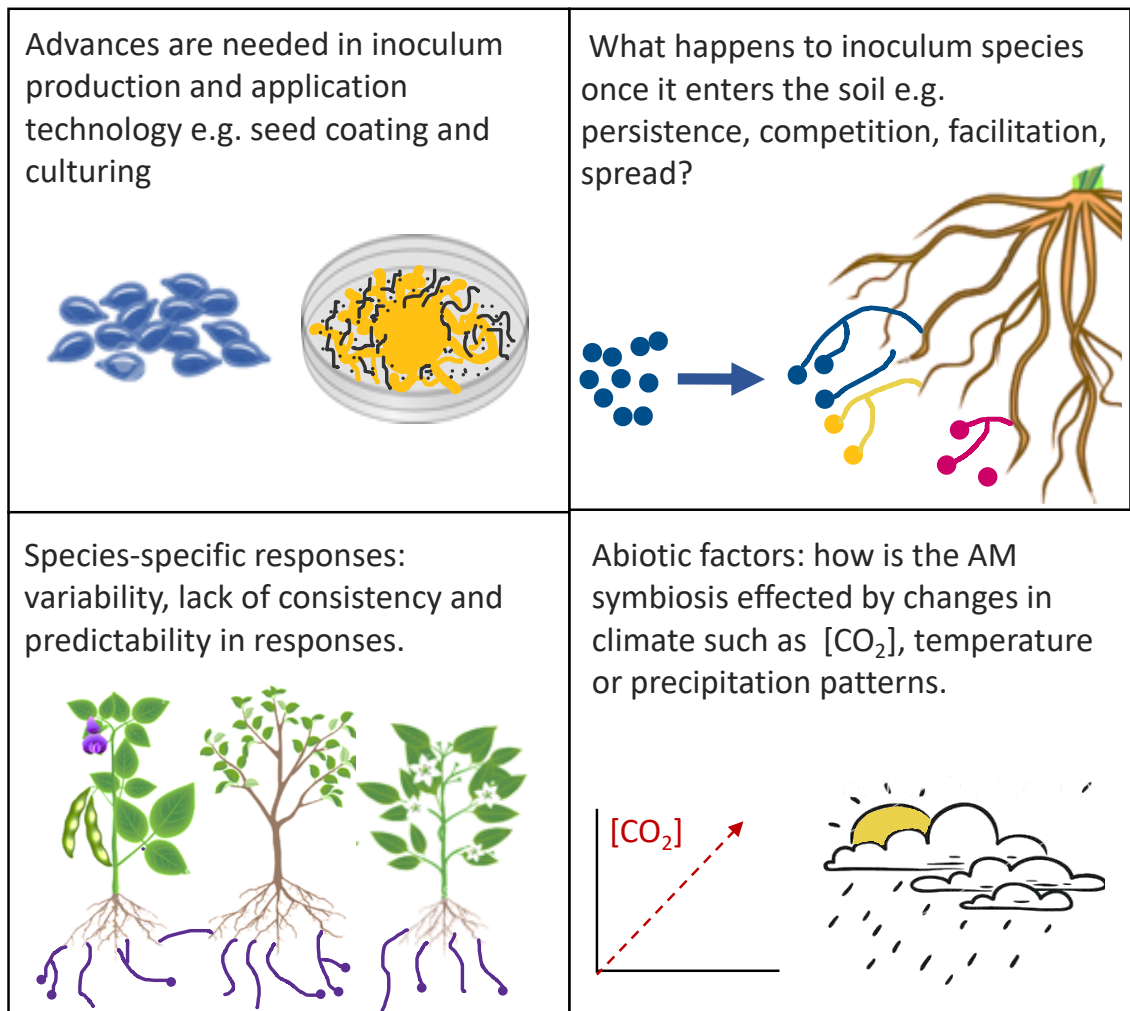
The inconsistency of crop-AMF interactions is one of the major barriers preventing the use of commercially produced AMF inoculum in agricultural systems. What determines the outcome of the symbiosis as positive, neutral or negative in terms of enhanced plant nutrient acquisition or growth is not currently clear. It is likely affected by a multitude of factors including biotic factors such as plant identity or the AMF community already present in the soil, and abiotic factors such as atmospheric [CO<sub>2</sub>] and temperature (Figure 5.2).

Climate change is likely to put extreme pressure on our food production system, through an increase in extreme weather events, temperature and pest pressures. Small increases in temperature and elevated [CO<sub>2</sub>] offer minor opportunities to increase crop yield, but only if nutrients and water are not limiting to crop growth. Increased application of chemical fertilisers is not viable to improve plant nutrient availability, and current research does not support an increase in nutrient acquisition via AMF at elevated [CO<sub>2</sub>]. Therefore, AMF's role in enhancing the CO<sub>2</sub> fertilisation effect is not clear, and studies should now be geared towards AMF's possible role mitigating yield losses associated with climate change.

In the future, combinations of abiotic factors should be studied together to give a more accurate prediction of how AM symbiosis could respond to future climates. Although this thesis focused only on rising atmospheric [CO<sub>2</sub>], global temperatures will also rise, with a predicted 4.5-6°C increase by 2100 (IPCC, 2017). Unlike [CO<sub>2</sub>], temperature changes can act directly upon the fungus as the root and fungus sometimes have contrasting growth patterns (Gavito et al., 2005). However, the fungus will also be affected indirectly through the plant host, which may allocate higher amounts of carbon below ground at higher temperatures, possibly due to increased fungal sink strength (Gavito et al., 2005). Yet, how AMF responds to temperature changes in terms of supplying AMF acquired nutrients

to their plant partners, especially crop plants, is unknown and could influence whether the use of commercial available AMF inoculum is advisable.

There is also evidence AMF associations could confer increased resistance to environmental stresses, including drought tolerance, and pest and disease resistance (Jung et al., 2012; Lehnert et al., 2018), all of which are predicted to become more problematic in the future (West et al., 2012; Peters et al., 2014; Rial-Lovera et al., 2017) .



**Figure 5.2:** The research and technological barriers to the use of commercially available AMF inoculum in agricultural systems. Technological advances are needed in inoculum production and application to drive down costs and make inoculation financially viable. More research is needed into how the outcome of the symbiosis is affected biotic factors such as plant identity or the AMF community already present in the soil, and abiotic factors such as atmospheric [CO<sub>2</sub>] and temperature.

The variation in responsiveness of wheat cultivars to AMF as seen in this study suggests future research could focus on breeding crops for their responsiveness to AMF. Many studies report significant differences among crop varieties in their response to mycorrhizal associations (Hetrick et al., 1993; Kaeppeler et al., 2000; Zhu et al., 2001), suggesting breeding programmes could capitalise on these genetic differences to select for material which exhibits positive responses to AMF associations (Fester & Sawers, 2011). If breeding made crop responses to AMF reliably positive, AMF inoculants could be used as a partial replacement of chemical fertilisers or to enhance crop growth and yields to maintain food security.

Before crop breeding for AMF responsiveness can be successful, we need to understand how genetic variability between wheat cultivars influences the function of plant-fungal interactions (Johnson et al., 2012). Recently, genome-wide associations studies (GWAS) have been used to identify quantitative trait loci (QTL) associated with wheat's ability to form symbiosis (Lehnert et al., 2017). Six QTL regions have been identified with possible candidate genes for marker-assisted selective breeding (Leiser et al., 2016). However, selecting for ability to form the symbiosis is not conducive to positive responses to the symbiosis. A further study employed the same approach to identify two genomic regions associated with plants response to AMF under drought stress, as AMF were found to significantly improve tolerance to drought in wheat (Lehnert et al., 2018). Further research and confirmatory studies in this area could lead to the inclusion of markers for response to AMF under drought stress in existing breeding programmes aiming to produce drought-tolerant crop varieties (Lehnert et al., 2018).

Alternatively, different AMF isolates may be beneficial in different crop cultivars and under different environmental conditions, including elevated [CO<sub>2</sub>] (Johnson et al. 2005). Therefore, changes in the diversity and dominance of a single species could affect the favourability of AMF associations. Pairing advanced community analysis techniques such as next generation sequencing, with functional studies, will be essential for the interpretation of any AMF community changes, on crop growth and nutrient acquisition. There is currently no consensus on what aspects of AMF diversity (e.g. richness or evenness) favour crop growth (Rodriguez & Sanders, 2014). Therefore, identifying community

changes associated with positive plant growth responses could inform future decisions about when/if commercial inoculum should be applied.

If researchers can bridge the gap between research and commercialisation, inoculant formulations could be produced, to engineer positive AMF responses in different crop cultivars and environments. For example, if technological advances such as reliable seed coating with AMF are achieved, then each new crop rotation could have a seed coating tailored to the crop type or environment without prohibitive costs of inoculation. Inoculant formulations could be created which are 'future climate-proof'. Critically, identifying species which generate yield increases and enhance nutrient acquisition under elevated [CO<sub>2</sub>] could be valuable as we aim to mitigate crop losses associated with climate change by capitalising on CO<sub>2</sub> fertilisation effects without exacerbating fertiliser use.

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